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Characterization of novel hypertrophie signaling pathways activated by the AKAP-Lbc signaling complex in cardiomyocytes

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Prof. Christian Widmann

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TABLE OF CONTENTS

ANNEXE : Article published in Journal of Biological Chemistry

RÉSUMÉ

L'hypertrophie cardiaque représente un mécanisme d'adaptation du myocarde en réponse à différents stress. Sur le long terme, l'hypertrophie cardiaque peut évoluer vers l'insuffisance cardiaque, l'une des principales causes de morbidité et de mortalité dans les pays industrialisés, pour cette raison, la communauté scientifique est très intéressée à élucider les voies de signalisation qui régulent ce phénomène pathologique dans le cœur.

Notre laboratoire a montré que AKAP-Lbc, une protéine d'ancrage de la protéine kinase A (AKAPs), est principalement exprimée dans le cœur et peut réguler des processus importants tels que l'hypertrophie des cardiomyocytes.

AKAP-Lbc fonctionne comme un facteur d'échange de nucléotides guanine (GEF) pour la petite Rho-GTPase RhoA. Cette fonction est activée par différents récepteurs qui activent son domaine Rho-GEF. Des études récentes ont démontré que AKAP-Lbc est impliquée dans la réponse hypertrophique des cardiomyocytes suite à l'activation des récepteurs α1-adrénergiques. Le but général de ce travail de thèse est la caractérisation de la voie de signalisation hypertrophique activée par AKAP-Lbc dans les cardiomyocytes.

Mes travaux montrent que AKAP-Lbc organise un complexe macromoléculaire, comprenant les protéines kinases PKN, MLTK, MKK3 et p38 et active la protéine kinase p38 en réponse à l'activation des récepteurs α1-adrénergiques.

Nos résultats indiquent que cette voie de signalisation au cours de la réponse hypertrophique active le facteur de transcription GATA4 et la protéine Hsp27.

GATA4 est un important facteur de transcription qui régule la transcription de plusieurs gènes au cours de la réponse hypertrophique, alors que Hsp27 est une protéine chaperonne qui interagit avec le cytosquelette des cardiomyocytes et les protége contre le stress hypertrophique.

Pris ensembles, ces études contribuent à comprendre comment le complexe de signalisation formé par AKAP-Lbc régule l'hypertrophie dans les cardiomyocytes. Au-delà de leur intérêt au niveau biochimique, ces travaux pourraient aussi contribuer à la compréhension du phénomène de l'hypertrophie dans le cœur.

ABSTRACT

In response to various pathological stresses, the heart undergoes a remodelling process that is associated with cardiomyocyte hypertrophy. Since cardiac hypertrophy can progress to heart failure, one of the major cause of lethality worldwide, the intracellular signalling pathways that control cardiomyocyte growth have been subjected of intensive investigation. While we have previously shown that AKAP-Lbc, an A-kinase anchoring protein (AKAP) with an intrinsic Rho specific guanine nucleotide exchange factor activity, is critical for activating RhoA and transducing hypertrophic signals downstream of the α1-adrenergic receptors (ARs), the hypertrophic pathways activated by this anchoring protein are poorly characterized. Here we show that AKAP-Lbc can assemble a novel transduction complex containing the RhoA effector PKNα, MLTKβ, MKK3β and p38α, which integrates signals from α1-ARs, to promote RhoA-dependent activation of p38α.

In particular, molecular disruption of the AKAP-Lbc/p38 complex in rat neonatal cardiomyocytes, reduces the ability of α1-AR to activate p38 and to induce cardiomyocyte hypertrophy.

It is well established that activated p38 can promote cardiomyocyte hypertrophy through the activation of different transcription factors and cytosolic proteins. Importantly, we could show that suppression of AKAP-Lbc expression in cardiomyocytes, inhibits α1-AR mediated activation of the hypertophic transcription factor GATA4 as well as the heat shock protein Hsp27, a protein that stabilizes sarcomeres and cytoskeleton during hypertrophic stress.

Altogether these results indicate that in cardiomyocytes AKAP-Lbc organizes a transduction complex that includes PKNα, MLTKβ, MKK3β and p38α, that promotes cardiomyocyte hypertrophy, potentially through the activation of GATA4 and Hsp27.

These findings provide a novel mechanistic hypothesis explaining how assembly of macromolecular complexes, that specify MAPK signaling, mediate cardiomyocyte hypertrophy downstream of α1-ARs.

ABBREVATIONS

AC	Adenylyl cyclase
AKAP	A-kinase anchoring protein
ANF	Atrial natriuretic factor
Ang-II	Angiotensin-II
AR	Adrenergic receptor
ATP	Adenosine triphosphate
ATR	Angiotensin II receptors
Brx	Breast cancer nuclear receptor auxiliary factor
α-CA	alpha-cardiac actin
C3	Clostridium botulinum C3 toxin
cAMP	Cyclic 3,5-adenosine monophosphate
CC	Coiled-coil region
cDNA	Complementary deoxyribonucleic acid
DAG	Diacylglycerol
Dbl	Diffuse B-cell lymphoma
DH	Dbl Homology domain
EPI	Epinephrine
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
ETR	Endothelin receptors
FSK	Forskolin
GAP	GTPase activating protein
GATA	GATA binding protein 4
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Ganine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GST	Gluthatione S-transferase
GTP	Guanosine triphosphate
HEK-293	Human embryonic kidney 293 cells

Hsp-27	Heat shock proteins 27
IP3	Inositol triphosphate
JIP	C-Jun N-terminal kinase)-interacting protein
JNK	C-Jun N-terminal kinase
LARG	Leukemia-associated Rho guanine nucleotide exchange factor
Lbc	Lymphoid blast crisis
LC3	Microtubule –associated light chain 3
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MEF2	Myocyte-specific enhancer factor 2
α-MHC	alpha-Myosin heavy chain
β-MHC	beta-Myosin heavy chain
MLK3	Mixed-lineage protein kinase 3
MLTK	MLK-like mitogen-activated protein triple kinase
MKK3	Mitogen-activated protein kinase kinase 3
mRNA	Messenger ribonucleotidic acid
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
PDE	Phosphodiesterase
PDZ	PSD-95, DLG, and ZO-1 domain
PE	Phenylephrine
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositol-3 kinase
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
PKD	Protein kinase D
PKN	Protein kinase N
PLC	Phospholipase C
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2 A
PP2B	Protein phosphatase 2 B / calcineurin
PRK	PKC-related kinase

RNVMs	Rat neonatal ventricular cardiomyocytes
RBD	Rho-binding domain
RGS	Regulator of G protein signaling
ROCK	Rho kinase
SH2	Src homology 2 domain
SH3	Src homology 3 domain
shRNA	Short hairpin ribonucleotidic acid
SRE	Serum response element
SRF	Serum response factor
α-Ska	alpha-skeletal actin
TAC	Transverse aortic constriction

I. INTRODUCTION

1.1. Cardiac Hypertrophy

The mammalian heart is a dynamic organ that can grow and change to accommodate alterations in its workload. In response to various biomechanical or neurohumoral stresses, the heart undergoes an adverse remodeling process that leads to the development of cardiac hypertrophy, a process characterized by the hypertrophic non-mitotic growth of cardiomyocytes, apoptotic cardiomyocyte death, activation and proliferation of fibroblasts and excessive deposition of extracellular matrix [1-3]. Hypertrophy can be induced both by biomechanical stresses including hypertension, myocardial infarction, ischemia associated with coronary artery disease, valvular insufficiency and stenosis and inherited mutation of genes encoding contractile proteins; as well as neurohumoral stresses induced by excessive release of hormones, cytokines and peptide growth factors at the level of the myocardium. Remodeling events associated with cardiac hypertrophy are maladaptive and ultimately contribute to depressed contractile function and progression to heart failure, a major cause of lethality worldwide.

Interestingly it is well described another form of hypertrophy named physiological cardiac hypertrophy. Physiological cardiac hypertrophy is a physiological response to exercise training and it differs from pathological hypertrophy in its structural and molecular profile and is characterized by normal organization of cardiac structure and normal or enhanced cardiac function [4] (Fig.1.1).

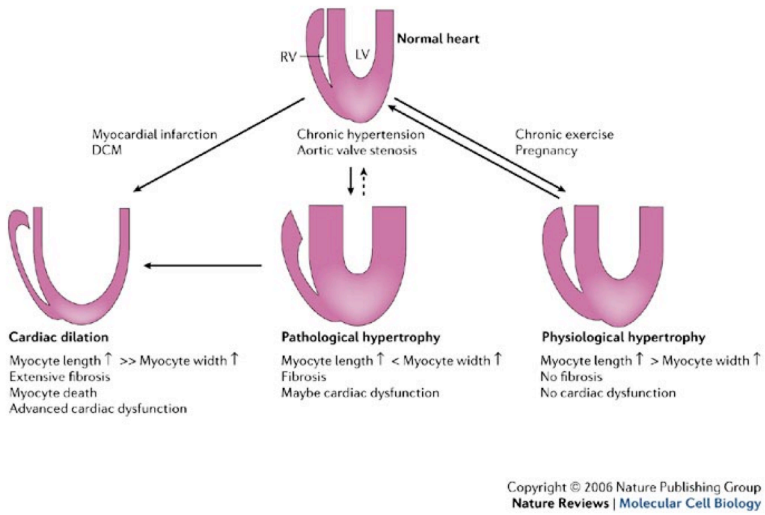


Figure 1.1: Progression of pathological cardiac hypertrophy: Pathological cardiac hypertrophy can produce concentric hypertrophy in which the ventricular wall and septum thicken with a net decrease in ventricular chamber dimensions. This remodelling is associated with a greater increase in cardiac myocyte width than length. However, pathological cardiac hypertrophy can also produce a phenotype of eccentric and dilatory cardiac growth. Cardiac dilation, although not typically referred to as hypertrophy, can result from a growth response in which sarcomeres are predominantly added in series to individual myocytes.

On the other hand, at the cellular level, the pathological cardiomyocyte growth is associated with a profound reorganization of the myofibrilles that constitute sarcomers and the upregulation of specific subsets of "fetal" genes that are normally expressed during embryonic life including sarcomeric proteins such as myosin heavy chain β, myosin light chain 2v and α-skeletal actin as well as several signal transduction proteins and transcription factors that directly control protein synthesis, calcium homeostasis and cardiomyocyte metabolism [5]. It is now clear that aberrant expression during adult life of these fetal proteins strongly affects cardiac contractility, calcium handling and myocardial energetics and leads to maladaptive changes in cardiac function [6].

At the molecular levels the most proximal initiating stimuli for cardiomyocyte hypertrophy can be broadly segregated into biomechanical and stretch-sensitive mechanisms, or neurohumoral mechanisms that are associated with the release of hormones, cytokines, chemokines and peptide growth factors.

All these different stimuli are sensed by cardiac myocytes through an array of different receptors that initiate the cardiac growth response by activating a circuit of signaling pathways that alter different genes expression (Fig. 1.2).

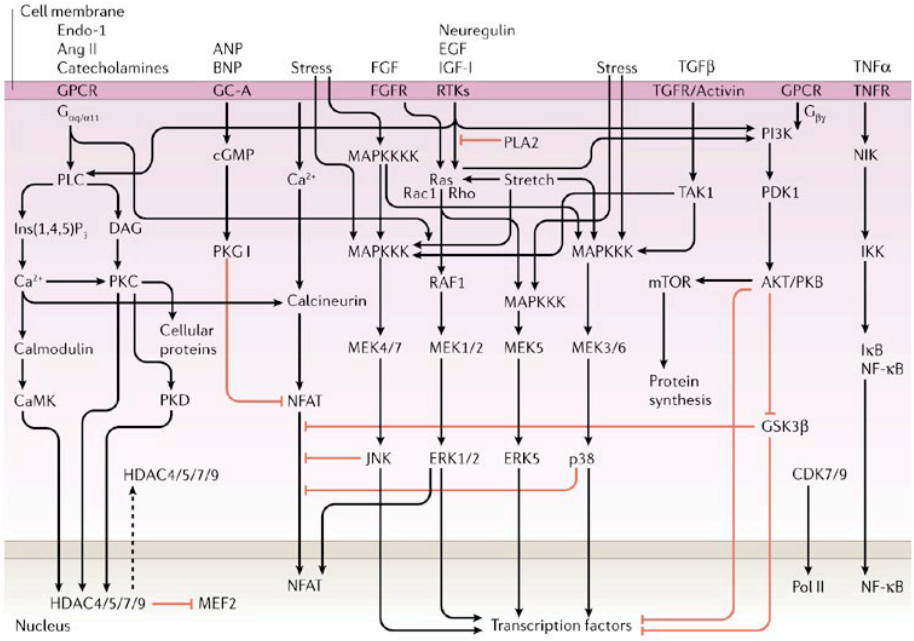


Figure 1.2 Signal-transduction pathways that coordinate the cardiac hypertrophic response: The most well characterized signaling pathway activated during the process of cardiac hypertrophy.

Most of these stimuli induce a first phase of the cardiac hypertrophy named compensatory hypertrophy, where it is activated the growth of each single cardiomyocyte, it is increased

the cardiac pump function and it is decreased the ventricular wall tension. Unfortunately, all these stimuli in the long term induce a second phase of the cardiac hypertrophy that predisposes to heart failure, arrhythmia and sudden death.

This second phase is characterized by a progressive deterioration of the left ventricular function that is related to a progressive loss of cardiomyocytes due to apoptosis. In addition to the cardiomyocyte apoptosis, progressive accumulation of cardiac fibroblasts in the heart occurs in the failing heart that may lead to ventricular diastolic or systolic dysfunction.

Since heart failure remains the main cause of mortality in the Western world, the investigations to understand the different pathways that regulate this complex pathology are becoming an important priority to the scientific community and to the health systems.

1.2. Sensing of hypertrophic signals by G protein coupled-receptors

Most stimuli, known to initiate pathological changes associated with the development of cardiac hypertrophy, are sensed by cardiomyocytes through an array of membrane-bound receptors that include mainly Receptor tyrosine kinases (RTK)s, Receptor Serine/Threonine Kinases (RSTK), Cytokine receptors and G protein-coupled receptors (GPCRs).

GPCRs constitute a family of over 800 genes encoding receptor proteins that are characterized by a seven-transmembrane (7TM) configuration. Members of this family include receptors for many hormones, neurotransmitters, chemokines and calcium ions, as well as sensory receptors for various odorants, bitter and sweet taste, and even photons.

Although several studies have demonstrated that this family of receptors can mediate their biological function through different signaling pathways the best characterized mechanism is the ability of these receptors to activate the heterotrimeric G proteins.

Heterotrimeric G proteins are the molecular switches that turn on intracellular signaling cascades in response to the activation of GPCRs by extracellular stimuli. Therefore, G proteins have a crucial role in defining the specificity and temporal characteristics of the cellular response. Heterotrimeric G proteins are composed of three subunits, α, β and γ, and their switching function depends on the ability of the G protein α-subunit to cycle between an inactive GDP-bound conformation that is primed for interaction with an activated receptor, and an active GTP-bound conformation that can modulate the activity of downstream effector proteins.

Upon agonist binding, the activated receptor induces the release of GDP from the α subunit of G protein that is replaced with GTP. This leads to dissociation of the G-protein complexes into α subunits and βγ dimer, which both activate several effectors. When the receptor is no longer active, the intrinsic GTPase activity of the Gα subunit hydrolyzes the GTP to GDP and the GDP-bound Gα subunit associates with Gβγ subunits. In this manner, the signaling cycle of the heterotrimeric G protein is complete.
There are four major families of heterotrimeric G proteins, based on their α subunits, named $G\alpha_s$, $G\alpha_I$, $G\alpha_q$, $G\alpha_{12}$, that mediate the activity of a variety of different effectors.

- Gs (stimulatory) - activates adenylate cyclase to increase cAMP synthesis.
- Gi (inhibitory) - inhibits adenylate cyclase.
- Gq - stimulates phospholipase C.
- G12 - stimulates the Rho-GTPase pathways.

In the cardiac system, GPCRs play important roles during the embryonic develop of the heart in response to physiological stimuli and during pathological alterations [7]. In particular three GPCRs coupled with Gq play different important functions in the myocardium: the α adrenergic receptors (α-AR), the endothelin A/B receptor (ET_AR; ET_BR) and the angiotensin 1/2 receptors (AT1R; AT2R).
The ability of these receptors to mediate hypertrophic cardiomyocyte growth was demonstrated in vitro using primary cultures of rat neonatal ventricular cardiomyocytes (RNVMs) as a model system, as well as in vivo using animal models over-expressing or lacking individual GPCRs [8].

Several lines of evidence have characterized the role of the AT1Rs in the development of cardiac hypertrophy in vitro and in vivo. It has been demonstrated that AT_1 mRNA expression increases in response to aortic coartation or acute myocardial infarction [9]. In addition, long-term treatment of stroke-prone spontaneously hypertensive rats with AT_1 blocker candesartan significantly reduced left ventricular mass [10]. Moreover, beneficial effects of AT_1 receptor blocker on left ventricular hypertrophy extend to patients with mild-to-moderate hypertension [11].

Similarly, it has been shown that stimulation of Endothelin receptors (ET_AR and ET_BR) cause cardiomyocyte hypertrophy in vitro and in vivo [12] and their expression is increased

in the hypertrophied [13] and failing heart [14]. On the other hand, it has been demonstrated the ET receptor antagonists attenuate the development of cardiac hypertrophy and heart failure [15]. These findings suggest that ETRs are involved in the development of cardiac hypertrophy and heart failure.

On the other hand, it is now appreciated that α1-ARs are also involved in the regulation of various pathological processes including vascular muscle cell hypertrophy, proliferation and migration in response to injury [16, 17] as well as cardiac hypertrophy [18, 19].
The activation of the α1-ARs in RNVMs is a classical model of cardiac hypertrophy that has provided much of the available information about cardiac growth signaling pathways. Experiments in cultured neonatal rat cardiac myocytes show clearly that catecholamines induce hypertrophy via α1-AR [20].
In transgenic mice different experiments partly support the culture results and the idea that α1-ARs can be sufficient to induce hypertrophy during development.
In particular, the activated mutant of the α1B-AR over-expressed in the heart amplified the hypertrophic response to pressure overload and also resulted in heart failure and premature death [18]. Similar results have been observed in transgenic mice that have a cardiac restricted over-expression of the WT α1B-AR given an infusion of phenylephrine (PE is a α1-AR agonist) [21].
In marked contrast, over-expression of α1A-ARs did not promote the cardiac hypertrophy in response to the thoracic aorta constriction (TAC) [22], in fact, heightened α1A-ARs activity improved survival [23].
These interesting and surprising results indicate that the pathological hypertrophy associated with pressure overload is influenced by α1B-ARs, whereas the physiological hypertrophy associated with postnatal growth may involve both receptor subtypes.
The hypertrophic pathways activated by αARs, ATIRs and ETRs have been associated with the activation of the two heterotrimeric G-proteins, Gq and G12/13 coupled to the receptors. Here below are summarized the main findings illustrating the importance of these two heterotrimeric G-proteins in the transduction of signals involved in cardiac hypertrophy.

1.2.1 Sensing the hypertrophic signals by the heterotrimeric G-proteins Gq

It is well established that the α subunit of the heterotrimeric G protein Gq plays a major role to mediate the effects of α1-ARs, ATIRs and ETRs on cardiac hypertrophy [24]. Furthermore, mice lacking Gαq/Gα11 in cardiomyocytes are protected from cardiac hypertrophy in response to pressure overload [25]. In addition, an important study from the group of Lefkowitz has shown that inhibition of Gq protein, by targeting the receptor-Gq interface, reduced the myocardial hypertrophy induced by pressure overload [26].

Different studies using genetically modified mouse models have confirmed that Gq/11 coupling is a necessary event in the induction of pathological cardiac hypertrophy. Gq/11, by activating phospholipase Cβ (PLCβ), induce the generation of diacylglycerol (DAG), which functions as an intracellular ligand for protein kinase C (PKC), leading to PKC activation, and production of inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) [8]. Accumulation of Ins(1,4,5)P3 leads to the mobilization of internal Ca^{2+} by directly binding to the Ins(1,4,5)P3 receptor located in the sarcoplasmic reticulum or the nuclear envelope. Liberation of internal Ca^{2+} stores can mediate the hypertrophic signaling through calcineurin–NFAT (nuclear factor of activated T-cells) activation or calmodulin-dependent kinase (CaMK)–HDAC (histone deacetylase) inactivation [27].

In line with these findings, a recent study has demonstrated that GPCRs coupled to Gq can induce cardiac hypertrophy by a novel ERK1/2 activation. Interestingly, this paper has clearly shown that Gαq activates the classical pathway of ERK1/2, while the βγ subunits released from Gq enhanced the autophosphorylation of ERK1/2 on its Threonine 188, which increases the efficiency of ERK1/2 activation [28].

While most of the studies have focused on the role of Gq in mediating the hypertrophic effects, recent evidences now suggest that G12 and G13 also contribute importantly to the growth responses initiated by α1-AR, ATIRs ETRs [29]. In fact, it is now appreciated that

functional inhibition of G proteins of the G12/G13 family can strongly impair receptor-induced cardiomyocyte hypertrophy [29].

1.2.2 Sensing of hypertrophic signals by the heterotrimeric G-proteins G12/13

As reported in the paragraph above, the α1-ARs, ATIRs and ETRs are coupled also to the heterotrimeric G proteins G12/13 [30]. Interestingly this family of G proteins includes two distinct α subunits, Gα12 and Gα13, that are responsible to regulate the activation of the Rho-GTPase through different RhoGEF proteins and modulate various cellular responses such as cytoskeletal changes and cellular growth [31]. However, despite their close similarity in sequences and some effectors, Gα12 and Gα13 have distinct roles in the cellular homeostasis.

In vitro, it has been shown that Gα13 is more potent to induce apoptosis [32], while Gα12 seems to play an important role to induce cardiomyocyte hypertrophy in response to α1-ARs, ATIRs and ETRs stimulation [33-35].
In particular, an important work has shown that the expression of RGS domain of p115RhoGEF, which blocks the activation of Gα12 and Gα13, inhibits JNK activation and the hypertrophic responses in RNVMs [33, 34]. Interestingly this study has demonstrated that the activation of Gq-mediated signaling pathway is not sufficient to induce the hypertrophy and it needs the concomitant activation of G12/13 to activate the cardiac hypertrophy.
This line of evidence has been shown also by our group in RNVMs where over-expressing the dominant negative mutant of Gα12 blocked cardiomyocyte hypertrophy induced by PE [36]. Collectively, all these data indicate the critical role of G12 family in vitro to activate hypertrophic signaling independently of Gq.

On the other hand an important study in vivo has demonstrated that activation of G12/13 in cardiomyocytes did not affect the cardiac hypertrophy but it induced the cardiac fibrosis in response to pressure over-load [37].

Altogether these findings highlighted the role of G12/13 to activate the Rho-GTPases and to induce the hypertrophic response of the heart. Here below are summarized the main findings illustrating the importance of Rho-GTPases in the transduction of signals involved in cardiac hypertrophy.

1.3 The small G-proteins and Rho family

Small molecular weight GTPases of the Rho family belong to the super-family of Ras-GTPase. In humans the Ras super-family comprises more than 150 members and it is well conserved during the evolution, where different Ras orthologs are present in animals, Drosophila, C. elegans, S. cerevisiae and plants.

The members of the Ras family are divided into five major branches on the basis of sequence and functional similarities: Ras, Rho, Rab, Ran and Arf. Although similar to the heterotrimeric Gα subunits in biochemistry and function, Ras family proteins function as monomeric G proteins. Small G-proteins cycle between an active GTP-bound state, which is able to bind effector proteins, and an inactive GDP-bound state. In this context they exhibit high-affinity binding for GDP and GTP, and possess low intrinsic GTP hydrolysis and GDP/GTP exchange activities.

Interestingly, there are three main classes of regulatory protein that control GDP/GTP cycling. The first one catalyzes the exchange of GDP with GTP by its guanine-nucleotide-exchange factor (GEF) domain [38], whereas the second one regulates the intrinsic GTPase activity of the small G protein to promote formation of the inactive GDP-bound by GTPase-activating protein (GAP) domain (Fig. 1.3) [39].

The third class of regulators includes Rho-GDIs (GDP dissociation inhibitors), that sequester the prenylated GDP-Rho-GTPases as cytosolic complexes and prevent the exchange of GTP with GDP on the Rho-GTPases.

Rho-GTPases are divided into 7 subgroups that include Rho, Rac, Cdc42, Rnd, RhoD, RhoH/TTF, RhoBTB and Miro. Currently the three best characterized members are RhoA, Rac1 and Cdc42 (Fig. 1.4).

It is now clear that Rho GTPases are involved in several actin-dependent processes such as migration adhesion, morphogenesis, axon guidance, and phagocytosis [40, 41]. In this context RhoA has been shown to promote actin stress fiber formation and focal adhesion

assembly; Rac1 lamellipodium formation and membrane ruffling; and Cdc42 formation of actin microspikes and filopodia [42].

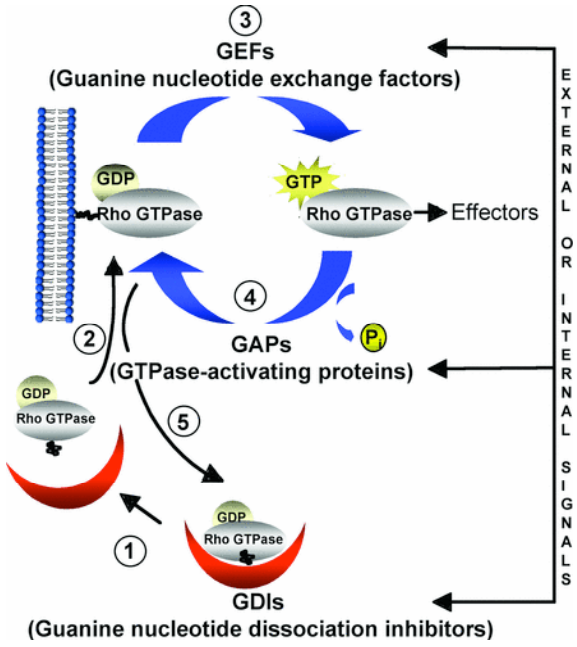


Figure 1.3 Small G-proteins GDP–GTP cycle : GDP–GTP cycle is tightly regulated by three families of proteins, including the GEFs, GAPs and GDIs In the absence of intracellular or extracellular signals, the Rho GTPases are maintained in the cytosol by the GDIs which mask their lipid modifications. External or internal cues promote their release from the inhibitory complex (1) that enables them to associate with the plasma membrane (2) where they are activated by GEFs (3) and can signal to effector proteins. Then, GAPs inactivate the GTPases by accelerating the intrinsic GTPase activity, leading to the GDP-bound form (4). Once again, the GDI molecules stabilize the inactive GDP-bound form in the cytoplasm, waiting for further instructions (5).

In addition to their roles in controlling the actin cytoskeleton, Rho-GTPases regulate cell polarity [43], gene expression [44], cell cycle progression [45], and membrane transport pathways [46].

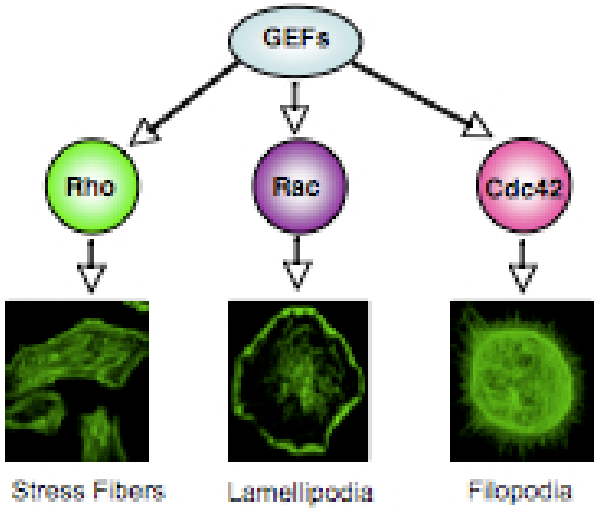


Figure 1.4 Rho family GTPase and actin cytoskeleton remodeling: The Rho Family GTPase Rho induces stress fibers, GTPase Rac induces lamellipodia and GTPase Cdc42 induces filopodia

In this introduction, I will focus on the different roles of Rho-GTPases to regulate the reorganization of cytoskeleton and to induce cardiomyocyte hypertrophy.

1.4 The small G-proteins signaling in cardiac hypertrophy

During the last decade two Rho-GTPase members, RhoA and Rac1, have been linked to agonist-induced cardiomyocyte hypertrophy in vitro. Moreover, the generation of different transgenic mice has demonstrated that RhoA and Rac1 signaling pathways have complex roles in inducing cardiac hypertrophy.

1.4.1 Rac1

It is well established that Rac1 has an important role in the induction of cardiomyocyte hypertrophy through different pathways.

The early study performed by the group of Finkel had shown that expression of constitutively active Rac1 (V12Rac1) in RNVMs resulted in an increased sarcomeric reorganization and cell size compared to the controls. In contrast, the expression of the dominant negative Rac1 (N17Rac1) resulted in attenuated morphological hypertrophy in response to hypertrophic PE stimulation. These results suggest that Rac1 is an essential element of the signaling pathway leading to cardiac myocyte hypertrophy [47].

Rac1 is well characterized to mediate its hypertrophic effects through mitogen-activated protein kinases (MAPKs). In this context, it has been demonstrated that PE induced cardiomyocyte hypertrophy through the activation of a pathway involving Rac1 and the extracellular regulated kinase ERK1/2 pathway [48].

Another study has clearly shown that mechanical stretch induces cardiac hypertrophy through the activation of a Rac1- reactive oxygen species (ROS)-p38 dependent pathway in cardiomyocytes [49].

In addition, it has been demonstrated that Rac1/ROS required the activation of the kinase ASK1, which in turn activates the transcription factor NF-kB, to induce cardiomyocyte hypertrophy [50].

On the other hand, the implication of Rac1 in cardiac hypertrophy in vivo cannot be studied by conventional gene targeting approaches, because the deletion of Rac1 gene results in embryonic lethality.

Nevertheless, transgenic mice that express the constitutively active Rac1 (V12Rac1) specifically in the myocardium have been created (these mice expressed the mutant form of Rac1 at comparable levels to the endogenous Rac1). Interestingly, the constitutive

activation of the Rac1 signaling pathway resulted in two distinct cardiomyopathic phenotypes: a lethal dilated phenotype associated with neonatal activation of the transgene and a transient cardiac hypertrophy seen among juvenile mice that resolved with the age of mice [51].
In line with these findings, a recent paper has demonstrated that conditional cardiomyocyte-specific Rac1 deletion prevents Ang II-induced cardiac hypertrophy [52].

In conclusion during the last decade different studied in vitro and in vivo have clearly shown a connection between Rac1 and cardiac hypertrophy.

1.4.2 RhoA

There are numerous data indicating that RhoA is required for cell transformation and proliferation in a variety of cell types. In particular, RhoA, the main Rho GTPase expressed in cardiomyocytes, has received particular attention in later years because of its crucial role in mediating hypertrophic responses.

Initial experiments performed using RNVMs revealed that inhibition of RhoA using dominant negative mutants of RhoA or the C3 botulinum toxin could strongly impair hypertrophic gene expression, protein synthesis, and myofibrillar reorganization induced by hypertrophic stimuli [53-55].
These early studies provided the evidence that, in vitro, RhoA is required for transducing growth signals downstream of hypertrophic stimuli. More in depth, these studies have demonstrated that receptors coupled to Gq and G12/13 including, α1-ARs, ATIRs and ETRs are able to induce the hypertrophic response through the activation of RhoA.
On the other hand, in vivo, two lines of research have provided indirect evidence for the implication of RhoA in cardiac hypertrophy.
The first study published more than ten years ago from the laboratory of Brown has characterized two lines of transgenic mice in which they expressed the constitutively activated RhoA (L63A) or wild type RhoA in a cardiac specific manner through the α-MHC promoter [56]. Interestingly most of the founders and progeny of RhoA L63A mice did not survive to adulthood while there was just an increased mortality of the mice that over-expressed the wild-type RhoA. Surprisingly, these mice did not develop any obvious left ventricular hypertrophic response, even if the two hypertrophic genes Atrial natriuretic

factor (ANF) and the beta Myosin heavy chain (β MHC) were more expressed compared to the controls.
In contrast, the transgenic mice had at typical phenotype of heart failure with a severe edema, ventricular chamber dilatation, increased cardiac fibrosis, atrial enlargement and decreased fractional shortening. Another important information given by this study was that some transgenic mice developed atrial fibrillation, atrioventricular block and a severe bradycardia. These effects can be explained by the roles of RhoA in modulating the activity of cardiac ion channels.
Recently, an important study coordinated by the group of Condorelli has shown that inhibition of RhoA expression in healthy cardiac tissue by the micro RNA (miR) 133 prevents activation of hypertrophic pathways and that down-regulation of miR 133 in response to pressure overload promotes an increase in RhoA expression and development of cardiac hypertrophy [57].

The concept that RhoA can promote the cardiac hypertrophy it was already suggested by studies showing that the inhibition of RhoA isoprenylation and membrane localization by statins could reduce the hypertrophy induced by mechanical stress or by GPCR agonists [58].

Based on this findings I will present below the current state of knowledge on the activation of RhoA and its implication on signaling pathways in cardiac hypertrophy.

1.5 Activation of Rho-GTPase by Rho-GEF proteins

With their 69 distinct homologues, RhoGEFs proteins represent the largest family of direct activators of Rho-GTPases in humans, and they activate Rho-GTPases within particular spatio-temporal contexts, in response to different extracellular stimuli.
All guanine nucleotide exchange factors that exhibit exchange activity towards Rho GTPases share a Dbl homology (DH) domain (named after Dbl, the first identified member of the family) and an adjacent pleckstrin homology (PH) domain [59, 60]. The DH/PH tandem domain is known as GEF domain. The DH domain interacts extensively with the switch regions of Rho GTPases and thanks to its nucleotide-exchange activity it catalyzes the exchanging reaction of GDP with GTP in Rho-GTPase. DH domains cause the

remodeling of the switch regions to alter the nucleotide-binding pocket. The induced alterations in the GTPase encourage GDP and Mg^{2+} dissociation. This event leaves the nucleotide-binding pocket completely exposed to allow rebinding of GTP-Mg^{2+} in cells [61].

The PH domain is located immediately distal to DH domains, and it cooperates for the activation of Rho GTPases, as several studies have demonstrated that DH–PH fragments show greater nucleotide-exchange activity as compared to DH domains alone [62].

This can be explained by the fact that PH domain can participate to the GTPase-binding interface as demonstrated for the Rho-GEF protein named LARG where the PH domain makes direct contacts with RhoA [63]. Another function of the PH domain is to target the RhoGEF at the plasma membrane where it can be positioned in proximity of Rho-GTPases allowing their efficient activation [64] [65].

The modulation of the GEF activity is the crucial event to activate the Rho-GTPases. In this context, different mechanisms have been characterized to regulate the GEF activity of different Rho-GEF proteins including the activation by GTP-binding protein, protein kinases and by the regulation of phosphoinositol kinases [60].

Several lines of evidence now suggest that Rho-GEFs can integrate signals from G protein-coupled receptors [60]. In particular the RhoA specific GEFs, p115 Rho-GEF, LARG and PDZ-Rho-GEF, AKAP-Lbc can be activated by the α–subunits of G12/G13 [66-68].

On the other hand, LARG and the newly identified p63-Rho-GEF can also integrate signals from the α subunit of Gq [69].

The best characterized mechanism to activate the GEF domain is through the GTP-binding protein Gα12/13. Interestingly, biochemical studies identified p115RhoGEF, LARG and PDZ-RhoGEF, as a subset of GEFs containing a regulator of G-protein signaling (RGS)-like domain, that is typically associated with enhancing the intrinsic GTPase activity of heterotrimeric Gα subunits. This interaction is responsible for the activation of the GEF domain. However, the activation of Rho-GEF by G12/13 often needs protein tyrosin kinases as regulators. For example, LARG and PDZ-RhoGEF can be activated by Gα12 after the phosphorylation by the two kinases FAK, (focal adhesion kinase) and Tec [67].

Interestingly, also Gq can activate Rho in PLCβ and PKC-independent manner. One of the best studies that validate this pathway had shown that in mouse embryonic fibroblasts the

Gq protein induced the stimulation of the Rho-GEF protein LARG, that in turn activated RhoA [70]. In addition a recent paper has determined the crystal structure of the Gαq-p63RhoGEF-RhoA complex, detailing the interactions of Gαq with the Dbl and pleckstrin homology (DH and PH) domains of p63RhoGEF. These interactions involved the effector-binding site and the C-terminal region of Gαq and appear to relieve autoinhibition of the catalytic DH domain by the PH domain [71].

The second important mechanism to activate the GEF domain is mediated by the phosphorylation of the Rho-GEF proteins by protein kinases. In this context the RhoGEF protein Vav1 has revealed that at the basal state its N-terminal region interacts with the DH domain to prevent access of the GTPases. Upon phosphorylation of Tyr174 by various receptor-associated tyrosine kinases, the N-terminal region becomes unstructured and releases the DH domain, that is able to activate the Rho-GTPases [72].
Finally, a third modulation mechanism involved in the activity of the GEF domain is mediated by the phosphoinositol kinase. In mammalian cells, early studies have shown that treatment with the PI3K inhibitor Wortmannin inhibited the activation of Rac downstream of growth factor receptor activation [73]. In addition, PtdIns(4,5)P_2, the substrate of PI3K, was found to inhibit substrate binding and GEF activity of the DH domain of Vav and Sos1 [74], while the PI3K product (PtdIns(4,5)P_3) binding to the PH domain of Vav and Sos1 displayed enhanced GEF activity.
Altogether these results support the hypothesis that phosphatidylinositol and the PI3K are important to modulate the activity of different GEFs proteins.

An important characteristic of Rho-GEFs is that they can organize pathways downstream of Rho-GTPases. This has been shown for the Rac-specific GEF Tiam1, which recruits, through the scaffold protein JIP 2, a signaling complex that transduces activating signals from Rac1 to the activation of p38 MAPK [75].
In addition, another Rho-GEF protein, named p115-Rho-GEF, is coupled with the scaffold protein hCNK1 (human connector enhancer of ksr), which organizes and regulates JNK pathway following RhoA activation (Fig. 1.5). Moreover, this study indicated that the macromolecular complex organized by hCMK1 is important to induce the stress-fiber formation and SRF activation [76].

Altogether these results have highlighted the role of Rho-GEFs as crucial proteins that transduce activating signals from the heterotrimeric G-proteins to the activation of the small Rho-GTPases. Moreover recent evidence has demonstrated that Rho-GEF proteins can act as scaffold proteins and organize macromolecular signaling complexes.

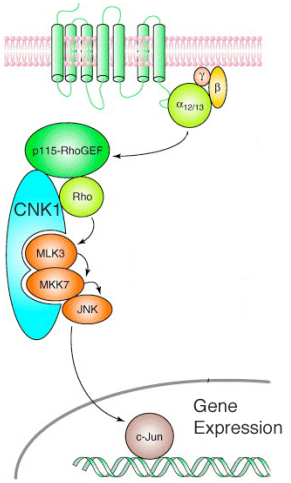


Figure 1.5: Scaffold proteins determine the signaling specificity of Rho-GTPase. Cell-surface receptors coupled to the heterotrimeric G proteins Gα12 and Gα13 activate the small GTPase Rho via RhoGEFs, including p115-RhoGEF The ability of the scaffold protein hCNK1 to form a molecular bridge between p115-RhoGEF, Rho, and the protein kinases MLK3 and MKK7, leads to the selective activation of JNK by Rho via this MAPK cascade. *Marinissen and Gutkind. Trends Biochem. Sci. 2005.*

1.6 Signaling pathways activated by RhoA during cardiac hypertrophy

Rho-GTPases mediate their cellular effects by interacting and activating target proteins, termed effectors, which preferentially associate with the active (GTP-bound) form of the GTPase. Several RhoA effectors are currently known, including three families of protein kinases, the Rho-activated kinases (ROCK1 and 2), Citron kinase and PKN-related kinases (PRK1 and PRK2), the myosin binding subunit of the myosin light chain phosphatase (MBS), as well as adaptor proteins such as Rhotekin, Rhophilin, Citron-N, Diaphanous homologues (Dia1, mDia2, and mDia3), and kinectin.
Interestingly, among these effectors, only ROCKs and PRK1 have been shown to play a role in transducing hypertrophic pathways downstream of RhoA.

1.6.1 ROCK

The first characterized effector of RhoA is ROCK, a serine/threonine kinase that serves as an important mediator of numerous cellular functions, including focal adhesion formation, motility, smooth muscle contraction, and cytokinesis. ROCK is found in two forms, ROCK

1 (ROCKβ; p160- ROCK) and ROCK 2 (ROCKα). Both ROCK 1 and ROCK 2 contain an amino-terminal catalytic kinase domain, a central coiled-coil domain of about 600 amino acids, and a carboxyl-terminal pleckstrin homology (PH) domain that is split by a cysteine-rich region. The C-terminal of ROCK is thought to inhibit the kinase activity by masking the kinase domain. Rho-GTP interacts with the C-terminal portion of the central coiled-coil domain and activates the kinase activity of ROCK [45].
ROCK promotes actin–myosin-mediated contractile force generation through the phosphorylation of numerous downstream target proteins. For example, ROCK phosphorylates LIM kinase-1 and kinase-2 (LIMK1 and LIMK2) at conserved threonines in their activation loops, increasing LIMK activity, and the subsequent phosphorylation of cofilin proteins, which blocks their F-actin-severing activity [77].

The implication of ROCK in cardiomyocyte hypertrophy has been shown in vitro and in vivo. Hypertrophic stimuli such as PE, AngII and ET-1 activate RhoA/ROCK pathway in RNVMs [53-55]. Interestingly blocking the ROCK activity in myocytes, using the specific inhibitor Y27632, reduced the activity of the transcription factors SRF and GATA4 that are known to mediate the hypertrophic response downstream of RhoA [78].
On the other hand, an important study indicates that ROCK1 haplo-insufficient mice did not show any decreased hypertrophy but rather decreased fibrosis in respect to the wild type mice [79].
Similarly, treating chronically hypertensive rats with two Rho kinase inhibitors, Y-27632 and fasudil [80], abolished fibrosis but not hypertrophy induced by hypertension.
Normally, inhibition of ROCK with Y-27632 can reduce infarct size and apoptosis resulting from 30 minutes ischemia followed by 24 hour of reperfusion. Interestingly, inhibition of Rho kinase prevented the ischemia/reperfusion-induced increase in proinflammatory cytokines, suggesting a possible role for RhoA/ROCK signaling in cardiac inflammation in vivo [81].

Altogether these findings indicate that activation of RhoA/ROCK signaling in the heart is deleterious. What is unclear from these studies is whether the observed salutary effect of blocking RhoA/Rho kinase signaling reflects changes in the cardiomyocyte or is a consequence of more directly inhibiting fibroblast proliferation/migration or of attenuating local inflammatory responses.

1.6.2 PKN

Protein Kinase N (PKN) is the first identified serine/threonine protein kinase activated by the RhoA. PKN is widely distributed in various organisms such as mammal, frog, fly, and starfish. There are at least three different PKN isoforms (PKNα/PAK-1/PRK-1, PKNβ, and PRK2/PAK-2/PKNγ) in mammals.

The general structure, common for all three isoforms (fig. 1.6), shows that in the C-terminal is placed a Ser/Thr type protein kinase domain which has high sequence homology to the one of PKC family members. The N-terminal region of PKN contains three tandem motifs of ~70 amino acids, each composed of a charged region followed by a leucine-zipper-like region. Structural analysis of the first repeat showed that it consists of two helices that form an anti-parallel coiled coil named ACC finger [82]. This domain is important because is responsible for the interaction with RhoA.

In all isoforms of PKN there is a stretch of about 130 amino acids between the ACC domain and the catalytic domain of PKN that has a weak homology to the C2 domain of PKC ε and η. The C-terminal part of the C2-like region functions as an auto-inhibitory region, which is sensitive to arachidonic acid (one of the activators of PKN) [83].

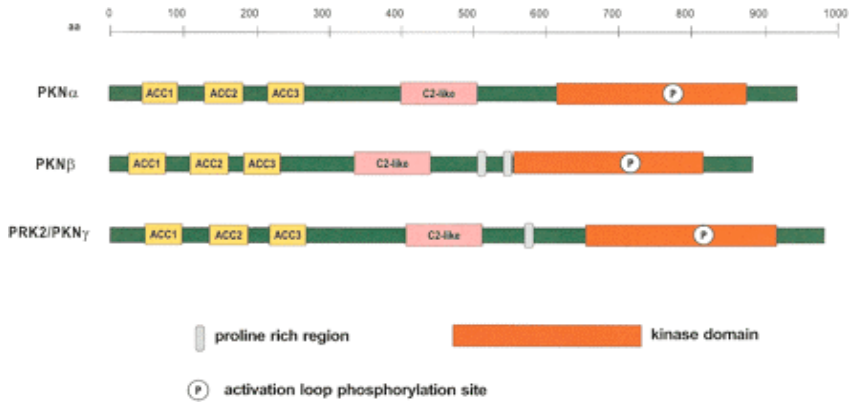


Figure 1.6: Primary structure of human PKN *Mukay, J. Biochem. 2003*

PKN has different effectors, which most of them include an SH3 domain such as phospholipase D1, α-actinin, and MLTK (Mixed lineage kinase-Like mitogen-activated protein Triple Kinase). Interestingly, it has been demonstrated that PKNα efficiently phosphorylates the kinase MLTKα, which has been recently identified as a MAPK kinase kinase (MAPKKK) for the p38 MAPK cascade. Phosphorylation of MLTKα by PKNα

enhances its kinase activity in vitro. Furthermore, PKNα associates with each member of the p38γ MAPK signaling pathway (p38γ, MKK6, and MLTKα). These results suggest that PKNα functions not only as an upstream activator of MLTKα but also a putative scaffold protein for the p38 MAPK signaling pathway [84].
In vitro, it has been demonstrated that PKNα stimulates actin stress fiber depolymerization and membrane ruffling in 3T3 L1 and Rat1-IR fibroblasts. The kinase-negative forms of PKNα (T774A or K644D) prevent insulin-induced actin stress fiber breakdown and membrane ruffling, suggesting that PKNα is involved in insulin-induced actin cytoskeletal reorganization [85].

In cardiomyocytes, PKNα acts downstream of RhoA to regulate the transcription of the hypertrophic transcription factor ANF through a serum response element (SRE), located in the responsive region of ANF, following the RhoA activation by PE stimulation [86].

In vivo, a recent study has demonstrated that the activation of PKN in the heart mediates the survival of cardiac myocytes during ischemia/reperfusion [87]. Interestingly this paper has clearly shown that activation of PKN by oxidative stress causes cardiac hypertrophy without left ventricular dysfunction. Moreover, this study demonstrated that PKN mediates its protective effect in part through the induction of the small Heat Shock Protein αB-crystallin (αBC), which in turn activates the ubiquitin-proteasome system.

In summary, in vitro experiments indicate that PKN contributes to the formation of stress fibers, to the activation of the p38 MAPK and to the expression of hypertrophic genes, whereas in vivo PKN is able to protect the cardiomyocytes from oxidative stress activating the ubiquitin-proteasome system mediated the activation of αBC.
The hypertrophic pathways linking PKN to the activation of p38 has been investigated during the last few years and will be discussed below.

1.7 Mitogen-Activated Protein Kinases and cardiac hypertrophy

The mitogen-activated protein kinase (MAPK) pathways are major signaling systems that transduce extracellular signals into intracellular responses such as growth, proliferation,

differentiation and apoptosis [88, 89]. MAPKs are proline directed serine/threonine kinases, which induce the vast majority of their physiological responses through the phosphorylation and activation of transcription factors [90]. Members of this protein kinase family regulate the expression of specific sets of gene and hence can mediate specific genetic responses to extracellular stimuli. Members of the MAPK family are activated by a protein kinase cascades in which the MAPK is phosphorylated and activated by a MAP kinase kinase (MAPKK) that is, in turn, phosphorylated and activated by a MAP kinase kinase kinase (MAPKKK) [88]. Frequently, a MAPKKKK kinase (MAP4K or MKKKK) activates the MAPKKK. The MAPKKKK or MAPKKK can be linked to the plasma membrane through a small GTPase or lipid. These MAPK cascades are organized into signaling complexes to create functional MAPK modules. Evidences collected over the past few years suggest that the organization of these modules are mediated by scaffolding proteins that interact with each of the protein kinases [91].

MAPKs are activated by dual phosphorylation of conserved threonine and tyrosine residues within the activation loop (denoted T-X-Y) and phosphorylate targets on serine and threonine residues within a consensus PXT/SP motif (X can depend on the MAPK).

On the other hand, the catalytic activity of MAPKs is attenuated by dual specificity MAPK phosphatases (MKPs), which dephosphorylates tyrosine and serine/threonine residues.

Active MAPKs frequently translocate from the cytoplasm to the nucleus to phosphorylate nuclear targets.

Pathway specificity is regulated at several levels, including kinase-kinase and kinase-substrate interactions, colocalization of kinases by scaffold proteins and localization to numerous subcellular structures such as: microtubules, endosomes, endoplasmic reticulum and actin cytoskeleton.

At present five families of MAPKs have been defined in mammalian cells: extracellular signal-regulated kinases (ERK1 and ERK2), Jun N-terminal kinases (JNK1, JNK2 and JNK3); p38 kinase isozymes (p38α, p38β, p38γ and p38δ); ERK3/ERK4; and ERK5 (Fig 1.7) [92]. The first three, and their activators, are the most known and characterized ones. They are implicated in development and in different human diseases, so for this reason are targets for drug development.

Among these kinases, ERK1/2 JNK, p38 and ERK5 have been described to play a role in the transduction of signals leading to cardiac hypertrophy.

Here below I will mainly focus on the role of ERK1/2, JNK and p38 kinases and highlight their importance in cardiac hypertrophy.

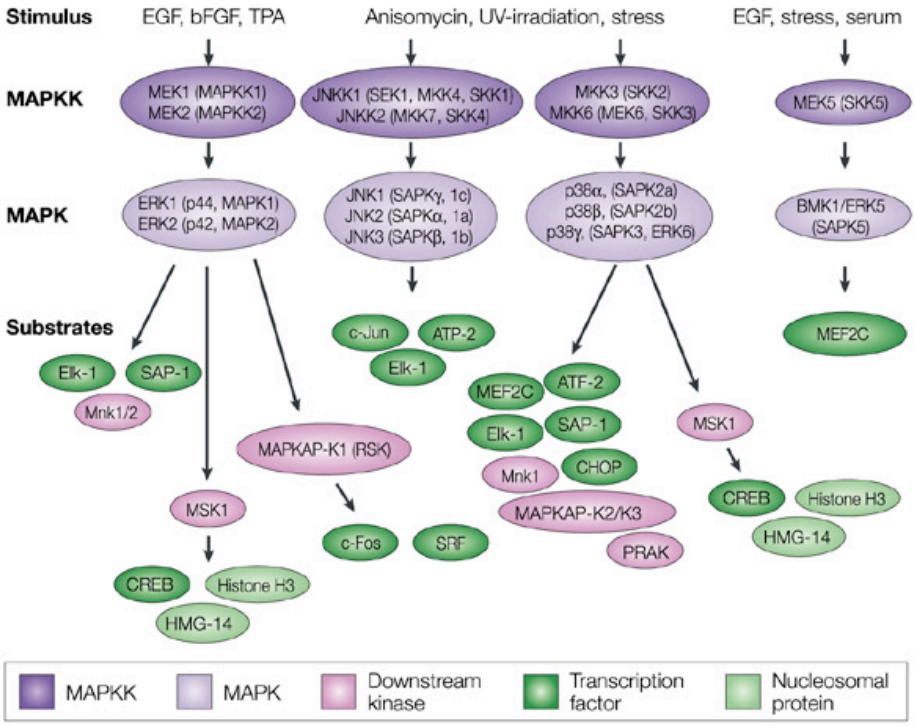


Figure 1.7: Schematic representation of mammalian MAPK cascades and target substrates, including transcription factors that are involved. *Nature Reviews Molecular Cell Biology 3, 30-40 (January 2002)*

1.7.1 ERK1/2

ERK1 and ERK2 are expressed in many tissues and form part of a MAPK module that includes the MAPKKK Raf (A-Raf, B-Raf, C-Raf/Raf-1) and the MAPKK MEK1/2. Ligands that bind the cell-surface-receptor tyrosine kinases or GPCRs, result in the activation of the small G-protein Ras by the Ras-GEF protein son of sevenless (SOS). Ras-GTP then triggers the activation of the MAPK cascade composed by Raf, MEK1/2 and finally ERK1/2.

In the heart, the ERK1/2 pathway is important during the heart development, and during the processes that lead to cardiac hypertrophy. Interestingly, during heart development ERK pathway plays several important roles in cardiomyocyte differentiation, cardiac morphogenesis, valve development and ventricular compaction [93].

The role of ERK1/2 in promoting cardiac hypertrophy has been extensively documented.

In particular, in vitro experiments performed on RNVMs have shown that ERK1/2 is a mediator of cardiomyocyte hypertrophy induced by the stimulation of α1-ARs [94], β-ARs [95] ATRs [96] and ETRs [97]. In line with these findings, overexpression of a constitutively active MEK1 mutant in RNVMs, leads to cardiomyocyte hypertrophy, while overexpression of dominant-negative MEK1 attenuates this response [98].
These findings have been partially confirmed in vivo. In fact transgenic mice expressing constitutively activated Ras (V-12-H-Ras) in cardiomyocytes developed pathological cardiac hypertrophy leading to sudden death [99]. In line with these results, the group of Muslin has shown that inhibition of the ERK1/2 pathway by overexpression in vivo the dominant negative Raf attenuated hypertrophy and fetal gene induction in response to pressure overload [100].
However, several studies also indicate that the ERK1/2 pathway can protect cardiomyocytes from apoptosis. In fact cardiac specific overexpression of dominant negative-Raf sensitized the heart to pressure overload and promoted cardiomyocyte apoptosis [100]. Similarly it has been reported that reduction of ERK1/2 activity leads to an increase in cardiomyocyte apoptosis without a significant impact on cardiac hypertrophy [101].

Recently, an important study has demonstrated that, upon dissociation from Gαq, the Gβγ subunit of Gq can interact with ERK1/2. This leaded to autophosphorylation of the kinase, its translocation to the nucleus, and the activation of pro-hypertrophic genes. This event is sufficient to induce cardiac hypertrophy and it has been reported in different failing human hearts [28].
In the same period another paper has demonstrated the role of ERK1/2 pathway in the regulation of cardiac fibrosis. In particular this study has shown that microRNA-21 (*miR-21*, also known as *Mirn21*) regulates the ERK pathway in cardiac fibroblasts, through the inhibition of sprouty homologue 1 (Spry1), that in turn negatively regulates the Ras/ERK pathway. Interestingly this paper has reported that in different human failing heart the levels of miR-21 and phosphorylated-ERK were increased whereas the level of Spry1 was dramatically decreased compared to the non-failing hearts [102]. In contrast a very recent paper of Olson has indicated that miR-21-null mice are normal and, in response to a variety of cardiac stresses, display cardiac hypertrophy, fibrosis, up-regulation of stress-responsive cardiac genes, and loss of cardiac contractility similarly to wild-type mice [103].

Altogether these results suggest that ERK1/2 has an important role to induce cardiomyocyte hypertrophy and to prevent cardiomyocyte apoptosis. On the other hand this pathway seems to be essential during the progression of cardiac fibrosis.

1.7.2 JNK

Members of the JNK family play crucial roles in regulating responses to various stresses, cellular development, inflammation, and apoptosis. There are ten isoforms derived from three genes: JNK1, JNK2 and JNK3. Interestingly JNK1 and JNK2 are expressed in many tissues while JNK3 is more restricted to the heart, brain and testis. At the molecular level JNK activation is induced by two MAPKKs named MEK4 and MEK7, which can integrate activating signals from at least 13 MAPKKKs including MEKK1-MEKK4, ASK and MLKs.

JNK cascades are organized by different scaffold proteins (including JIP1, JIP2, JIP3/JSAP1, JIP4, β-arrestin 2, filamin and CrkII) that presumably target the JNK modules to different sites in the cell and play roles in kinase activation and/or substrate selection [104].

The role of JNK in the heart and particularly in cardiac hypertrophy is not clear because there are conflicting results among the experiments performed in vitro and in vivo.

Initial in vitro studies have shown a potential role of the JNK pathway to induce hypertrophy. For example, the over-activation of JNK by constitutively active MKK7 led to a hypertrophic phenotype [105]. Likewise the dominant negative MKK4 was able to block the ET-1-induced hypertrophy of cultured myocytes [106]. Interestingly the same group has confirmed this last result in vivo, by over-expressing the dominant negative MKK4 in the adult rat heart by adenovirus-mediated gene transfer. This study indicated that overexpression of dnMKK4 reduced cardiac hypertrophy induced by pressure over-load [107]. In addition, another study has shown that cardiac specific inactivation of MEKK1 (the MAPKKK upstream of MKK4 and MKK7) blocked Gq-induced hypertrophy [108].

In contrast, several additional studies in vivo did not support this hypothesis. In particular, it has been shown recently that cardiac-specific deletion of MKK4 increased cardiac hypertrophy in response to pressure overload and in response to β-adrenergic stimulation [109]. Similarly the activation of JNK by overexpressing constitutively active MKK7 in the heart of transgenic mice did not induce cardiac hypertrophy [110].

Finally, an important study performed by the group of Rockman has investigated the role of the three different JNK isoforms in pressure overload induced cardiac remodeling. In this study they have generated three different cardiac specific knockout mice for the JNK1, JNK2 and JNK3. In response to pressure overload all three JNK knockout mice developed cardiac hypertrophy similar to wild-type mice (WT). [111].

In summary the role of JNK pathway in cardiac hypertrophy is still controversial with clear contrast results obtained in vitro, using cultured cardiomyocytes showing a pro-hypertrophic role of the JNK pathway, and in vivo using animal models showing an anti-hypertrophic role of the JNK pathway. This discrepancy perhaps reflects the complexity of signaling network involved in this process.

1.7.3 p38

The p38 MAPK (p38) family constitutes a second family of MAPK that are activated by numerous physical and chemical stresses, such as: UV irradiation, ischemia, cytokines (interleukin-1 and tumor necrosis factor), osmotic shock and heat shock.
Currently four isoforms of p38 including p38α (MAPK14), p38β (MAPK11), p38γ (MAPK12/ERK6) and p38δ (MAPK13/SAPK4) have been identified.
The p38α and p38β isoforms are expressed ubiquitously in adult tissues while the expression of p38γ is restricted mostly to skeletal muscle, and p38δ is enriched in lung, kidney, pancreas, placenta, and testis.
Structurally the p38-MAPK is composed of two domains: a 135-residue N-terminal domain that is composed largely of β-sheets and a 225-residue C-terminal domain that contains the catalytic site, magnesium binding sites, and phosphorylation sites [112]. The catalytic site lies at the junction between the two domains [113], and the common docking domain is located towards the C-terminus and is involved in the specific binding of upstream activators, substrates and phosphatases [114].
All the p38 kinases have the Thr-Gly-Tyr (TGY) dual phosphorylation motif, which is phosphorylated by upstream p38 activating kinases and corresponds to threonine (Thr180) and tyrosine (Tyr182).

Numerous MAPKKKs participate in the activation of p38 MAPK pathway, including ASK1 (Apoptosis signal-regulating kinase 1), kinases belonging to MLK (Mixed-lineage kinases)

family and Tak1 (TGF-beta-activated kinase 1). The MLKs regulate both p38 and JNK signaling pathways. Based on the domain arrangements and sequence similarity, MLK members are divided into three subgroups including: the MLKs, the dual-leucine-zipper-bearing kinases (DLKs), and zipper sterile-alpha-motif kinase (ZAK) (table 1).

In contrast, only three MAPKKs have been characterized to directly activate p38, including MKK3, MKK6 and MKK4. The MKK6 is able to activate all 4 isoforms of p38, whereas MKK3 preferentially phosphorylates the isoforms α and β. [115]. MKK4 was originally identified to be an upstream kinase of JNK, however subsequent studies have shown that it can also promote the activation of p38α and p38δ [116].

It is currently believed that p38 MAPK modules, containing distinct combinations of MAPKK and MAPKKK, can be assembled by specialized scaffolding proteins including JIP1, JIP2 and PKN [75, 84, 117]. Interestingly, among these scaffolds, PKN is the only one that is expressed in cardiomyocytes.

An interesting study suggested that PKN might represent a key-signaling enzyme that transduces signals from activated RhoA down to p38. In particular this study has shown that PKNα can assemble and organize a signaling module that includes MLTKα, MKK3/6 that promotes the activation of p38γ [84].

Subfamily	Synonyms	Production in mammals
MLK		
MLK1	/	Epithelial cells
MLK2	MST	Brain, skeletal muscle, testes.
MLK3	SPRK1 PTK1	Widely produced
MLK4α	/	Unknown
MLK4β	/	Unknown
DLK		
DLK	ZPK MUK	Brain, Keratinocytes, regenerating liver
LZK	/	Widely produced
ZAK		
ZAKα	MRK	Widely produced
ZAKβ	MLTK MLK7 ZAK	Widely produced

Table 1: Nomenclature and synonyms of mixed lineage kinase (MLK)

p38 can also be activated in a MKK-independent manner by two non-canonical pathways. The first one is mediated by the interaction of p38 with TAB-1 (Transforming growth factor β Activated kinase 1 binding Protein-1) that in turn leads the autophosphorylation and activation of p38 [118]. The second requires the phosphorylation of p38 on Tyr 323 by the T cells receptor-activated tyrosine kinase Zap70 (Zeta-chain-associated protein kinase 70). This event promotes subsequent autophosphorylation of p38 on residues Thr180 and Tyr182 [119].

Once activated, p38 can mediate its effects in the cytoplasm by activating substrates such as the MAP kinase-activated protein kinase 2 (MK2), MAP kinase interacting kinase1 (MNK1) lymphocyte-specific protein 1 (LSP1), Na+/H+ exchanger isoform, keratin 8, exc, or by activating nuclear substrates such as different transcription factors (ATF1/2/6, CHOP, p53, GATA4, MEF2A/C, ELK1, NFAT exc) [120].

The different effector proteins activated by p38 signaling pathways indicate that this protein kinase can regulates different cellular processes such as:

- Inflammation: A strong link has been established between the p38 pathway and inflammation. For example the activation of p38 plays essential roles in the production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) [120]
- Apoptosis: Abundant evidences exist for the involvement of p38 in apoptosis [121]. In particular it seems that p38α has a strong role to induce apoptosis. This finding was clearly shown by that overexpression of the constitutively activated MKK3, which increased the apoptosis of RNVMs. This apoptotic effect was suppressed by co-expression of the dominant negative p38α [122].
- Cancer development: It has been reported that p38 activation may be reduced in tumors and that loss of components of the p38 pathway such as MKK3 and MKK6 results in increased proliferation and likelihood of tumorigenic conversion regardless of the cell line or the tumor induction agent used [123] On the other hand there are several findings that indicate an important role of p38 on cell proliferation [124].
- Cell differentiation: p38α and p38β have been implicated in cell differentiation for certain cell types for example differentiation of 3T3-L1 cells into adipocytes [125]. Interestingly, different studies support the hypothesis that p38 regulates the differentiation of human embryonic stem cells into cardiomyocytes [126, 127].

- Cell cycle: The participation of p38α in cell growth has been observed in both yeast and mammals. For example G1 arrest of NIH3T3 cells caused by microinjection of Cdc42 was found to be p38α-dependent [128]
- Development: p38 has been linked to placental angiogenesis and erythropoietin expression suggesting a role in erythropoiesis [89]
- Cardiac hypertrophy: During progression of hypertrophy, both p38α and p38β levels were increased even if the 2 isoforms appear to have different effects that will be discussed below.

p38 signaling in cardiac hypertrophy

The implication of p38 isoforms in cardiomyocyte hypertrophy was initially suggested by in vitro studies performed in primary cultures of RNVMs using pharmacological inhibitors of p38. In particular, it has been shown that inhibition of the p38 pathway using the pharmacological inhibitor SB203580 was able to reduce hypertrophic cardiomyocyte growth in response to phenylephrine stimulation [129]. Likewise, the same study has shown that over-expression of the constitutively active MKK6, which specifically activates p38, was able to induce hypertrophy in absence of any hypertrophic stimuli.

These findings are in contrast with other results showing that the p38 inhibitor, SB203580, had no effect on ET-1-induced hypertrophy [106].

The in vivo evidence of p38 in cardiac hypertrophy is even more conflicting. In fact some studies clearly showed that cardiac-specific overexpression of dominant negative p38 either had no effect on hypertrophy or sensitized the heart to hypertrophy in response to pressure overload [130, 131].

Similarly, a recent study reported that transgenic-mice over-expressing in the heart either constitutively active MKK3 and MKK6 did not develop cardiac hypertrophy, but instead interstitial fibrosis. These mice die prematurely due to cardiac failure [132].

In contrast other studies suggest a prohypertrophic role of p38. This is the case in mice with the cardiac-specific inducible expression of constitutively active MKK3 that led to cardiomyocyte hypertrophy, cardiac remodeling and contractile dysfunction. Interestingly, the hypertrophic effects of MKK3 required MK2-dependent up-regulation of ciclo-oxigenase-2 (COX2). While COX-2 has been involved in the pathology of heart failure, the role and mechanism whereby COX-2 controls remain to be characterized. Furthermore, in this study it has been demonstrated that removal of the MK2 gene, that is one of the most

characterize p38 effectors, rescued some aspects of this pathological response, including partially ameliorated hypertrophy and contractile dysfunction, and prevention of early lethality. These beneficial effects were partially the consequence of the increased-protein synthesis of the MK2 [133]. Unfortunately the exact role of COX-2 in cardiac physiology and disease remains controversial even if during the last years different studies reported that specific COX-2 inhibitors significantly increased the risk of heart disease in chronic users [134].

These conflicting results can be explained by the fact that in heart the two main isoforms of p38 can promote different functions. In this context it seems that p38α (the predominant isoforms) plays role in cardiomyocyte apoptosis while the p38β seems to promote cardiomyocyte hypertrophy [122, 129, 135, 136].

Taken together these results indicate that the role of p38 in cardiac hypertrophy is a conflicting one. It seems that an acute activation of p38 is pro-hypertrophic while a chronic activation can lead to suppression of hypertrophic growth.

On the other hand recent findings have reported an interesting role of p38 in physiological hypertrophy. In particular an important study has shown that mice with a cardiac-specific deletion of ASK1 and a conditional cardiac knockout of p38 had an increased cardiac hypertrophy without fibrosis in response to swimming [137]. Partially in contrast, another study has demonstrated that transgenic mice over-expressing the p38 dominant-negative in response to swimming did not show any increased cardiac hypertrophy as compared to the wild-type mice [138].

Moreover, in the same study a transgenic mice line that over-expressed the dominant-negative protein 14-3-3 (that is a regulatory protein for p38) has shown a maladaptive hypertrophy with increased fibrosis and cardiomyocyte apoptosis in response to swimming [138]. As expected these mice had an increased p38 activity.

However, it is clear from both in vitro and in vivo studies that p38 activation has a detrimental effect on cardiac function and normal gene expression. Therefore, p38 induction is more closely related to pathological form of hypertrophy than to physiological compensation.

1.8 The p38 effector proteins activated during cardiomyocyte hypertrophy

1.8.1 Transcription factors activated by p38

In cardiomyocytes, p38 has been shown to promote the activation of the hypertrophic transcription factors GATA4 and MEF2.

GATA transcription factors are characterized by the conserved double zinc fingers that are required for binding to the specific consensus DNA sequence (A/T)GATA(A/G). Among the six GATA transcription factors in vertebrates, GATA4, GATA5, and GATA6 are expressed in the heart. Interestingly, GATA4-cardiac specific knockout in mice resulted in embryonic lethality with strong morphological defects of the heart indicating an important role of GATA4 during cardiac embryonic morphogenesis [139].

Functional analysis of the cis-regulatory elements has revealed that GATA4 directly regulates expression of cardiac-specific genes, such as: α-myosin heavy chain (α-MHC), β-myosin heavy chain (β-MHC), myosin light chain 1/3 (MLC1/3), cardiac troponin C, cardiac troponin I, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), cardiac sodium-calcium exchanger (NCX1) exc [140].

Consistent with the different genes regulated by GATA4, several studies have demonstrated that GATA4 plays an essential role in the activation of the fetal gene program in response to hypertrophic stimulation.

The functional activity of GATA4 is regulated through direct serine phosphorylation by the p38 and ERK1/2 following the stimulation of GPCRs, including α1ARs and AT1Rs [141-143].

In this context, it has been shown that activation of α1ARs in RNVMs by PE stimulation, activates a signaling pathway, which includes RhoA and p38 that increase the GATA4 transcription activity. In addition this study has identified for the first time that GATA4 is a RhoA/p38 mediator in sarcomere reorganization and hypertrophic gene regulation [141].

During the same period, the group of Sasayama demonstrated that the RhoA/ROCK pathway is linked to PE-induced GATA4 activation through the ERK pathway [55].

These findings demonstrate that regulation of GATA4 activity by the Rho/ROCK-ERK pathway promotes myocardial cell hypertrophy.

In addition, to phosphorylation the activity of GATA4 can also be regulated by interaction with transcriptional co-activators such as p300 and through the interaction with different transcription factors including: SRF, NFAT, FOG2, Nkx2-5 and MEF2 [144].

Taken together these results suggest that, hypertrophic stimuli positively regulate through multiple signaling pathways the GATA4 transcriptional activity. They also indicate that GATA4 plays an essential role in modulating the transcriptional regulation of subset of genes during the hypertrophic response.

On the other hand MEF2 family are the other important class of transcription factors activated by p38 during the cardiac hypertrophy.

In vertebrates, 4 members of MEF2 family (MEF2-A, MEF2-B, MEF2-C, and MEF2-D) have been identified. All the isoforms are expressed in many cells types and promote transcriptional regulation in the immune system, neurons, and striated muscle [145].

The two isoforms MEF2A and MEF2C appear to be more expressed in the postnatal mouse heart. Interestingly, targeted disruption of MEF2C has been shown to affect the morphological development of the heart during embryogenesis. Several cardiac genes including atrial natriuretic factor (ANF), cardiac α-actin, α-myosin heavy chain and myosin light chain (MLC)–1A expression have been shown to be down-regulated in MEF2C-null embryos, indicating an essential role of MEF2 in myocardial cell differentiation [146]. Similarly transgenic mice overexpressing dominant-negative MEF2C in the heart displayed attenuated postnatal growth of the myocardium [147].

MEF2 specifically binds the consensus DNA sequence CAT(A/T)4TAG/A and, consistently, to the A/T-rich DNA sequences that have been identified within the promoter regions of a number of cardiac genes, including α-MHC, MLC1/3, MLC2v, skeletal α-actin, sarcoplasmic reticulum Ca^{2+}-ATPase, cardiac troponin T, cardiac troponin C, cardiac troponin I, desmin, and dystrophin [148].

MEF2C is upregulated during the cardiac hypertrophy [147]. Interestingly, it has been reported that in the hypertrophied heart p38 phosphorylates and activates MEF2C but unfortunately the patho-physiological relevance of this event is not fully understood [129, 148].

Like GATA4, the MEF2 activity is regulated by several other proteins including the protein kinase ERK5[149], Ca^{2+}/CaM-dependent protein kinases (CaMKs), histone deacetylases (HDACs) [150], MyoD [151], NFAT [152], and GATA4 [153].

Altogether these results suggest that MEF2 and GATA4 are able to activate the transcription of several cardiac genes, such as ANP, BNP, α-MHC and α-cardiac-actin, indicating a cooperative role of MEF2 and GATA4 in the transcriptional regulation of these cardiac genes. However, it remains to be determined whether the transcriptional synergy between MEF2 and GATA4 is implicated in the generation of cardiac hypertrophy.

1.8.2 Non-genomic pathway activated by p38

As already reported before, p38 is able to activate a downstream protein kinase named MK2 [154] [155].

The mRNA of MK2 is expressed at detectable levels in most tissues, while at the protein level two different isoforms of MK2 have been described.
MK2 phosphorylates a variety of substrate proteins including heat shock proteins (Hsp27, αB-crystallin), enzymes that interact with the cytoskeleton (CapZ-IP, LSP1), mRNA-binding proteins (hnRNP), transcription factors (SRF), and regulators of the cell cycle and apoptosis (Cdc25C, BAG). Phosphorylation of these substrates by MK2 regulates the different cellular processes, such as the reorganization of the cytoskeleton, cell migration, cell-cycle regulation, chromatin remodeling and apoptosis [156].
In the heart, two different studies have shown a potential adverse effect of MK2 in response to stress stimuli. The first shows that MK2 cardiac-specific knockout mice were resistant to ischemia reperfusion injury [157]. Similarly, a second study indicates that cardiac-specific KO of MK2 in mice partially protects the heart from cardiac hypertrophy, interstitial fibrosis and contractile dysfunction induced by the acute activation of the p38 pathway [133]. Altogether these studies in vivo suggest that MK2 is a potential target of intervention for cardiomyopathy.
At the molecular level, one of the well characterized substrates of MK2 is the small chaperone protein Hsp27. Heat shock proteins (Hsps) are a family of highly conserved proteins which are activated by many cellular stresses and most of them function as chaperone proteins.
Hsp27 is member of the small heat shock protein family (HSPB) and displays a molecular weight of 27 kDa, but it can form large aggregates of around 800 kDa in the cytosol [158]. The Hsp27 mRNA is highly expressed in several tissues such as breast, uterus, cervix, placenta, skin, lung, heart and platelets. It has been involved in different biological

functions including cell migration, inhibition of apoptotic regulation, protection against oxidative stress, and modulation of inflammation [159]. The best characterized functions of Hsp27 are the ability to modulate actin polymerisation and to protect filaments degradation interacting with cytoskeleton components such as actin, intermediate filaments and microtubule [160].

In vitro it has been shown that in response to stress stimuli Hsp27 can be phosphorylated by different kinases including MK2/3/5, PKA and ASK1, that increased its chaperone activity, whereas in vivo studies have demonstrated that the phosphorylated-Hsp27 is able to stabilize actin filaments [161, 162].

In particular, a recent study reported that in human endothelial cells the phophorylation of Hsp27 regulates the actin microfilament dynamics and cell migration [163]. Similarly, others studies on endothelial cells pointed out that a heat shock induces an association of Hsp27 with stress fibres [164].

Collectively these results suggest that, following a stress, phosphorylated Hsp27 associates with actin filaments and stabilizes them.

Nevertheless, during the last years different studies indicate that Hsp27 has a direct anti-apoptotic function in response to stress stimuli.

In this context a recent study in monocytes has demonstrated that Hsp27 during apoptosis is able to interact with caspase-3 and inhibit its apoptotic activity [165].

In agreement with this anti-apoptotic effect, an important finding has clearly shown that Hsp27 regulates the p53 activity and blocks the cellular toxicity, induced by the doxorubicin treatment, in cardiac H9c2 cells. Overall this study has demonstrated that the anti-apoptotic effect of Hsp27 was mediated by an up-regulation of p21, which prevents the cell death [166].

In addition, a very recent paper has demostrated that Hsp27 protects adenocarcinoma cells from UV-induced apoptosis by the increased Akt stability and subsequently a prolonged p21-activation pathway that improved the survival of the cells [167].

Altogether these findings demonstrated that Hsp27 has a strong link with the anti-apoptotic function.

During the last five years different studies have begun to characterize the role of Hsp27 in cardiac phisiology.

An early study reported that the p38/Hsp27 pathway is involved in the differentation of the cardiomyocytes [126]. Interestingly, in xenopus Hsp27 is critical in the development of cardiac and skeletal muscle tissues [168].
On the other hand, some studies have demonstrated that over-expression of Hsp27 can protect cardiomyocytes against ischemic injury. In this context, Gaitanaki et al. have reported that the phosphorylated Hsp27 has a cardio protective role in isolated perfused amphibian heart, in response to oxidative stress [169]. Interestingly, different studies in vivo have suggested that Hsp27 can relocalize to the sarcomere in response to different stress stimuli, such as ischemic preconditioning and heat shock [170, 171].

Currently there is not evidence indicating a potential role of the pathway p38-MK2-Hsp27 in cardiac hypertrophy. It will be challenging to understand the functional role of this pathway in cardiac hypertrophy.

1.9 The A-kinase anchoring protein family

The cyclic AMP-dependent protein kinase (PKA) is a ubiquitous broad specificity serine/threonine kinase that controls a variety of cellular functions including metabolism, gene transcription, channel conductivity, cell growth, cell division [172]. It consists of a heterotetramer formed by two catalytic subunits held in an inactive state by association with a regulatory (R) subunit dimer. Binding of cAMP to the R subunits induces the dissociation and the activation of the catalytic subunits, and results in the phosphorylation of local substrates [172]. Stimulation of cardiac β-adrenergic receptors by norepinephrine (NE) released by sympathetic nerve terminals promotes PKA activation *via* a signaling pathway that includes the α subunit of the heterotrimeric G protein Gs and adenylyl cyclase (AC). Activated PKA plays a key role in modulating several cardiac functions including cardiac contractility, action potential duration and cardiac hypertrophy. At the cellular level, specificity of PKA action is achieved by controlling the subcellular localization of the kinase through the interaction of the regulatory subunits with A-kinase anchoring proteins (AKAPs), a group of functionally related proteins, which can be classified on the basis of their ability to associate with the PKA holoenzyme inside cells [173]. By positioning PKA in proximity of substrate targets and of cAMP gradients, which are generated at discrete

cellular microdomains by the opposing action of ACs and phosphodiesterases (PDEs), AKAPs ensure accurate substrate phosphorylation [174].

All members of the AKAP family share a conserved PKA anchoring domain which consists of a conserved amphipatic helix of 14-18 residues that interacts with an hydrophobic groove in the docking/dimerization (D/D) domain located in the extreme N-terminus of the regulatory subunit dimer [175, 176]. Compartmentalization of individual AKAP-PKA complexes occurs through specialized protein-protein or protein-lipid targeting domains located on each anchoring protein [173]. Another fundamental role of several AKAPs is their ability to coordinate other signaling enzymes such as kinases, phosphatases, phosphodiesterases, GTPases and other regulatory proteins into multivalent transduction complexes [174]. Finally, recent evidence demonstrates that AKAPs can form even larger macromolecular complexes by interacting directly or through adaptor proteins with upstream activators and/or with downstream PKA substrates [174]. Therefore, AKAPs can be considered as transduction units that ensure integration and processing of multiple signals to coordinately regulate the phosphorylation and function of specific cellular substrates.

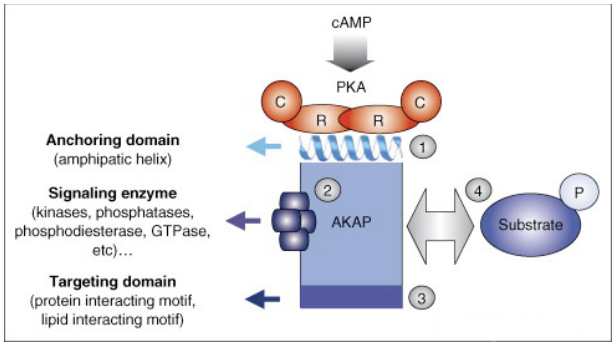


Figure 1.9 Functional motifs of AKAPs : (1) A conserved binding domain interacts with the AKAP binding surface on the regulatory subunit dimer of PKA. (2) Unique targeting domains direct AKAP signaling complexes to distinct intracellular locations. (3) Additional binding sites for other signaling components such as kinases, phosphatases, or potential substrates (4) Activation of substrates by PKA.

1.9.1 AKAPs in cardiac physiology

Different AKAPs are expressed in cardiomyocytes including Gravin, Troponin T, AKAP2, AKAP9 (Yotiao), mAKAP, AKAP18α/δ, AKAP95, AKAP79, AKAP121, AKAP220, AKAP250, AKAP350 and AKAP-Lbc, however a clear functional role has been established for a few of them [177].

In this context, AKAP9 (Yotiao) can form a complex with the potassium channel KCNQ1, which controls the myocardial duration of the action potential by carrying outward the K^+ [178].
Interestingly, Yotiao anchors the PKA responsible for the phosphorylation serine 27 and activation of KCNQ1. It interacts also with the protein phosphatase 1 (PP1) which is responsible for the de-phosphorylation the KCNQ1 at serine 27 inactivating the channel [179].

At the level of the sarcolemma, AKAP79 and Gravin interact with β adrenergic receptor (β1/2 for AKAP79, β2 for Gravin). These interactions increase the PKA phosphorylation of the receptors, that in turn regulates desensitization, internalization and recycling of the β AR [180].
AKAP18α, another AKAP present in the sarcolemma regulates the open probability of the L-type Ca^{2+}channel, by PKA phosphorylation. This event is critically involved in cardiomyocyte contraction because increasing the open probability of the channel causes an influx of Ca^{2+} in the cytosol that activates the ryanodyne receptor (RyR2). Once the RyR2s are activated they release Ca^{2+} from the sarcoplasmic reticulum (SR) into the cytosol, thus promoting the cardiac contraction.
Interestingly, the open probability of RyR2 is positively controlled by PKA phosphorylation of the inhibitory protein FKBP12.6 that is in turn regulated by mAKAP [181].
After cardiac contraction, the concentration of Ca^{2+} in the cytosol has to decrease to allow the next contraction. The largest amount of Ca^{2+} in cardiomyocytes is stored in the SR, and at this level AKAP18δ plays an important role in the re-uptake the Ca^{2+} back to the lumen of SR.
AKAP18δ interacts directly with phospholamban, which is a regulatory protein of SERCA (sarco/endoplasmic reticulum calcium ATPase). In response to β adrenergic stimulation, PKA anchored on AKAP18δ phosphorylates phosholamban that is not able to block the activity of SERCA [182].

Altogether these findings have shed new light on the role of AKAPs in heart pathophysiology. We will selectively discuss the role of AKAP-based transduction units in coordinating signaling pathways that control cardiomyocyte hypertrophy.

1.9.2 AKAPs in cardiac hypertrophy

mAKAP

Recent studies have shown that mAKAP, a large anchoring protein of 255-kDa highly expressed in the heart, can be targeted to the nuclear envelope of cardiomyocytes. At this location, mAKAP integrates and transduces several hypertrophic signals, as shown by the fact that silencing of mAKAP expression strongly reduces hypertrophic gene transcription induced by isoproterenol, phenylephrine and the leukemia inhibitor factor (LIF) [183, 184]. At the molecular level, it is now demonstrated that anchoring of PKA at the nuclear envelop through mAKAP favors β-AR-induced phosphorylation and activation of RyR2 [185]. The consequent increase in perinuclear calcium is thought to activate calcineurin Aβ which is bound to mAKAP [183]. This would results in the dephosphorylation and translocation of the pro-hypertrophic transcription factor NFAT to the nucleus whether it can activate hypertrophic gene transcription [186].

Additional biochemical studies have revealed that mAKAP directly interacts with the phosphodiesterase PDE4D3, which can recruit the pro-hypertrophic kinase ERK5 and its upstream activator MEK5 [184]. Since activated ERK5 can phosphorylate and activate the hypertrophic transcription factor MEF2c, one could raise the attractive hypothesis that, by positioning ERK5 in proximity of the nucleus, mAKAP could contribute to the activation of MEF2c-regulated hypertrophic genes.

AKAP121

AKAP121 assembles a multivalent signaling complex that includes PKA, the tyrosine phosphatase PTPD1, and src (proto-oncogene from avian sarcoma) on the outer membrane of mitochondria, and this plays an essential role in mitochondrial respiration. AKAP121 is widely expressed in different tissues and cell types, including cardiomyocytes [187].

Recent findings point to an important role in the regulation of the cardiomyocyte hypertrophy.

In this context it has been shown that silencing of AKAP121 in RNVMs induced a pronounced hypertrophy whereas the over-expression of AKAP121 reduced the cardiomyocyte size and impaired hypertrophy induced by isoprotenerol [188]. Interestingly, this study has shown that a reduction of AKAP121 expression is associated with a strong

de-phosphorylation and nuclear localization of the NFATc3 transcription factor. Moreover, it has been demonstrated by co-immuno-precipitation experiments that AKAP121 interacts with the phosphatase calcineurin, which is the up-stream activator of NFATc3.
Altogether these results identified AKAP121 as a negative regulator of cardiomyocyte hypertrophy.

1.10 AKAP-Lbc

We and others have identified a novel member of the AKAP family mainly expressed in cardiac tissues, termed AKAP-Lbc (AKAP13), that functions as a type II PKA anchoring protein as well as guanine nucleotide exchange factor (GEF) for the RhoA-GTPase [189, 190]. RhoA-activation is mediated by consecutive Dbl (DH) and pleckstrin homology (PH) domains that are located in the C-terminal half of the anchoring protein [189].

A truncated form of AKAP-Lbc missing both N- and C-terminal regulatory sequences, called Onco-Lbc, was originally identified as an oncogene from myeloid leukemia patients [191]. This oncogenic protein displays constitutive Rho-GEF activity and is able to induce cell transformation in a Rho-dependent manner [192].
Another splice variant of AKAP-Lbc, called Brx, has been identified in testis, estrogen-sensitive tissues, and immune cells [193]. In lymphocytes Brx-activating p38 signaling pathway leads to the activation of the NFAT5 messenger RNA that promotes the production of cytokines and induces inflammation [194].
AKAP-Lbc is the longest isoform with 2817 ammino-acids and it is expressed predominantly in the heart while in other tissue such as lung, placenta, kidney, pancreas, skeletal muscle and liver its expression is very low. Interestingly AKAP-Lbc is also expressed in different cell lines including HEK293, NIH3T3 and HeLa cells.
Our group has previously shown that Rho-GEF activity of AKAP-Lbc is strongly stimulated by the over-expression of the constitutive active Gα12 as well as by the activation of GPCRs coupled to Gα12 such as α1-ARs, ATIRs and LPA receptors (Fig. 1.10)
These findings place AKAP-Lbc in the growing family of Rho-specific exchange factors, including LARG, PDZ-RhoGEF and p115-RhoGEF, which relay signals from heterotrimeric G12/13 proteins to RhoA.

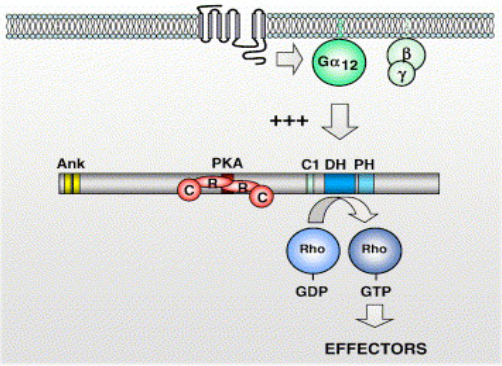
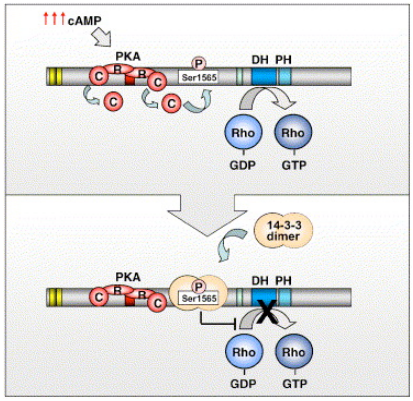


Figure 1.10 Activation of AKAP-Lbc: Activation of AKAP-Lbc occurs in response to agonists that stimulate G proteins coupled receptors linked to the heterotrimeric G protein G12. The Gα12 activates the guanosine exchange factor located in the region 1922-2337 of AKAP-Lbc which in turn switch on the small G protein RhoA. RhoA, through its effectors, regulates the organization of actin cytoskeleton and in the heart it is important during the process of cardiac hypertrophy

Evidence collected over the last ten years clearly indicates that PKA as a profound antagonistic effect on Rho signaling [195]. It is now appreciated that activation of the PKA holoenzyme anchored to AKAP-Lbc inhibits the Rho-GEF activity of the anchoring protein and blocks the activation of RhoA [195, 196].

Figure 1.11 Inactivation of AKAP-Lbc: ↑[cAMP] activates the PKA localized on AKAP-Lbc. PKA promotes the phosphorylation of AKAP-Lbc on serine 1565. This phosphorylation event induces the recruitment of a 14-3-3 dimer, which inhibits the Rho-GEF activity of AKAP-Lbc



Activated PKA phosphorylates the anchoring protein on serine 1565. This induces the recruitment of the regulatory protein 14-3-3, which inhibits the Rho-GEF activity of AKAP-Lbc [195, 196] (Fig. 1.11). The inhibitory effect of 14-3-3 is also dependent upon the oligomerization state of AKAP-Lbc as well as the anchoring of PKA to the complex [197]. In this context, a second mechanism involving the ubiquitin-like protein LC3 regulates the GEF activity of AKAP-Lbc. Interestingly, binding of LC3 to AKAP-Lbc strongly reduced the ability of the anchoring protein to interact with RhoA, profoundly impairing the Rho-GEF activity of the anchoring protein and, as a consequence, its ability to promote cytoskeletal rearrangements associated with the formation of actin stress fibers [198].

During the last years several studies have demonstrated that AKAP-Lbc is able to act as a scaffold protein that organizes different multiprotein complexes that mediate the activation of numerous signaling pathways.

In this context, recently, the group of John Scott has reported that in HEK cells AKAP-Lbc and the scaffolding protein kinase suppressor of Ras (KSR-1) form a signaling complex that efficiently relay signals from Raf, through MEK, and on to ERK1/2 [199].

In addition the group of Podolsky has demonstrated that AKAP-Lbc mediates the NF-κB activation following the Toll Like-receptors induction (TLR2). Moreover this study has demonstrated that NF-κB activation depends on the GEF function of AKAP-Lbc and that AKAP-Lbc knockdown reduces TLR2-induced JNK phosphorylation but not ERK1/2 and p38 [200].

In cardiomyocytes, AKAP-Lbc activation occurs following stimulation of α1-ARs via the α subunit of the heterotrimeric G protein G12 [189, 201]. Interestingly, silencing AKAP-Lbc expression in RNVMs strongly reduces both α1-AR-mediated RhoA activation and hypertrophic responses [201]. This suggested that this anchoring protein participates in a transduction pathway activated by the α1-ARs that includes Gα12, AKAP-Lbc and RhoA that promotes cardiomyocyte hypertrophy. It is currently unknown which Rho effector molecules mediate the hypertrophic effect of AKAP-Lbc in cardiomyocytes. Possible candidates could include ROCK [202] PKN [203] and stress-activated protein (SAP) kinases [29], which have been shown to control the transcription of genes involved in cardiomyocyte hypertrophy downstream of RhoA.
Recently, Carnegie and colleagues have reported the very interesting finding that AKAP-Lbc assembles a multiprotein complex that mediates the activation of protein kinase D (PKD) [204]. AKAP-Lbc contributes to PKD activation in two ways: first it recruits both PKD and its upstream activator kinase protein kinase Ch (PKCh), which phosphorylates and activates PKD in response to the activation of Gq-coupled GPCRs. Second, it facilitates the release of activated PKD into the cytosol, which occurs when serine 2737 within the PKD binding site of AKAP-Lbc is phosphorylated by anchored PKA [204]. Since PKD has recently been shown to play an important role in activating hypertrophic gene transcription via the regulation of chromatin-modifying enzymes [205, 206], one could raise the intriguing hypothesis that the released PKD could also contribute to the growth responses mediated by AKAP-Lbc. In fact they have demonstrated that PKD phosphorylates the histone deacetylase HDAC5 promoting its nuclear export. This event is crucial to increase

the activity of MEF2 transcription factor, which activates the hypertrophic response of RNVMs [207].
Based on these findings, one could speculate that interfering with the ability of AKAP-Lbc to activate Rho and/or to interact with PKD might represent a potential interesting strategy to reduce cardiac hypertrophy.

Recent evidence indicates that in mice, expression of AKAP-Lbc is strongly induced in the heart at the onset of cardiac hypertrophy in response so several hemodynamic or neurohumoral stresses such as thoracic aortic constriction, chronic infusion of angiotensin II or phenylephrine [36]. High AKAP-Lbc expression is subsequently maintained during the transition from hypertrophy to heart failure. This raises the hypothesis that AKAP-Lbc might play a role in the pathological cardiac remodeling process in vivo. AKAP-Lbc Knock out mice die in utero between 8.5 and 10.5 days post because of defects in heart tube formation [208]. While this argued for a role of AKAP-Lbc in cardiac development, it does not preclude the possibility to analyze the implication of the anchoring protein in the process of cardiac hypertrophy and heart failure.

So far, only few Rho-GEFs have been identified in cardiomyocytes. Interestingly, two sarcomere-associated Rho-GEFs, termed obscurin and p63Rho-GEF, have been proposed to control the organization of the sarcomeric cytoskeleton in RNVMs [209, 210].
These studies indicate that Rho-GEF proteins have a potential role in the organization of sarcomeric cytoskeleton, however it is not understood which pathways are activated by Rho-GEFs in response to hypertrophic stimuli.

II AIM OF THE THESIS

In response to numerous pathologic stimuli, the myocardium undergoes a hypertrophic response characterized by increased myocardial cell size and activation of fetal cardiac genes.
Our laboratory has previously shown that AKAP-Lbc is critical for transducing hypertrophic signals downstream of α1-ARs. While it is established that the effects of AKAP-Lbc on cardiomyocytes hypertrophy are mediated by the small molecular weight GTPase RhoA, it is currently unknown how hypertrophic signals are transmitted downstream of RhoA.

In the first part of my thesis, I have characterized at the molecular level a newly identified signaling complex organized by AKAP-Lbc that is critical for activating the p38α MAPK downstream of α1-ARs.

In the second part of my thesis, I have determined the implication of AKAP-Lbc/p38 activation complex in cardiomyocyte hypertrophy. In particular I have characterized novel hypertrophic pathways activated by the AKAP-Lbc signaling complex in primary cultures of rat neonatal cardiomyocytes.

III MATERIALS & METHODS

Expression Constructs

AKAP-Lbc fragments encoding amino acids 1585-1922, 1625-1922, 1655-1922, 1715-1922, 1765-1922, 1388–1585, 1388-1655, 1388-1715, 1388-1765 were PCR-amplified from the AKAP-Lbc pEGFPN1 vector [189] and subcloned at EcoRI/SalI into pFLAG-CMV6 vector to generate protein fragments fused with the Flag epitope. A region encoding residues 1388-1922 of AKAP-Lbc was PCR-amplified from the AKAP-Lbc pEGFPN1 vector and subcloned at SalI/NotI into pET30a vector to generate protein fragments fused with the histidine-tag. The AKAP-Lbc fragment encoding residues 1585-175 and corresponding to the PKNα binding domain of AKAP-Lbc was PCR-amplified from the AKAP-Lbc pEGFPN1 vector and subcloned at EcoRI/SalI into pFLAG-CMV6 and pEGFPN vectors to generate protein fragments fused with the Flag epitope and GFP, respectively.

The Flag-tagged AKAP-Lbc mutant missing the PKNα binding domain was generated by deleting the region encoding amino acids 1585-1715 by standard PCR-directed mutagenesis using the Flag-AKAP-Lbc vector [42] as a template.

Double-stranded hairpin (sh) oligonucleotides based upon the human AKAP-Lbc mRNA sequence (GI: 15986728, bases 6688-6706 and 228-246) were cloned into the HindIII and BglII sites in the pSUPER vector. The following oligonucleotides sequences were used: human AKAP-Lbc shRNA1 (sense strand) 5’ GTGCGTCTCAATGAGATTT 3’; human AKAP-Lbc shRNA2 (sense strand) 5’ GGTCAGTTCTGATACATTG 3’.

To generate lentiviral transfer vectors encoding AKAP-Lbc shRNAs, cDNA fragments containing the H1 RNA polymerase III promoter as well as the sequences encoding shRNAs were excised using BamHI/SalI from the pSUPER vector and subcloned into the pAB286.1 transfer vector. Mission® lentiviral transfer vectors encoding PKNα shRNAs or a control non-target shRNA were purchased from Sigma. These vectors contain a puromycin cassette that allows the selection of infected cells. The lentiviral packaging vectors pCMVDR8.91 and pMD2.VSVG encode the viral capsid and the vesicular stomatitis virus-G envelope protein, respectively [204].

Double-stranded hairpin oligonucleotides based upon rat (GI: 109462150, bases 6533-6551) AKAP-Lbc mRNA sequence were cloned into the HindIII and BglII sites in the pSUPER vector. The following oligonucleotides sequences were used: rat AKAP-Lbc shRNA (sense strand) 5'-GCAAGTCGATCATGAGAAT-3'; mutated rat AKAP-Lbc shRNA (sense strand) 5'-GCATGTCGATCATGCGATT-3'. Underlined base pairs in the mutated shRNAs are different from the wild-type AKAP-Lbc shRNAs. To generate lentiviral transfer vectors encoding AKAP-Lbc shRNAs, cDNA fragments containing the H1 RNA polymerase III promoter as well as the sequences encoding shRNAs were excised using EcoRI/KpnI or BamHI/SalI from the pSUPER vector and subcloned into the pSD28-GFP or pAB286.1 transfer vectors, respectively (1). The pSD28-GFP vector contains a GFP cassette under the control of a CMV promoter and was used to titer rat AKAP-Lbc shRNAs in HEK-293 cells.. On the other hand, the vector. pAB286.1 vector contains a puromycin cassette that allows the selection of infected cells and was used to titer rat AKAP-Lbc shRNAs in HEK-293 cells. Both vectors were used to express rat AKAP-Lbc shRNAs in primary cultures of rat cardiomyocytes. The lentiviral packaging vectors pCMVDR8.91 and pMD2.VSVG encode the viral capsid and the vesicular stomatitis virus-G envelope protein, respectively [204].

The full-length cDNA encoding human p38α was PCR amplified from a human heart cDNA library and subcloned at Not1-BamHI into pFLAG-CMV6, BamHI-XhoI into HA-pRK5 or BamHI-Not1 into pGEX4T1 to generate proteins fused to the Flag and HA epitopes or GST, respectively. Similarly, the full-length cDNA encoding human MLTKβ was PCR amplified from a human heart cDNA library and subcloned at NotI-BamHI into pFLAG-CMV6, BamHI-SalI into HA-pRK5 or BamHI-XhoI into pGEX4T1. Fragments 1-305 and 305-942 of PKNα were amplified from Myc-PKNα (generous gift from Dr. S. Gutkind, NIH, Bethesda, USA) and subcloned at Not1-SalI and NotI/XhoI into pET30a, respectively, to generate fusion proteins with the histidine-tag. HA-tagged JNK1, MKK3, MKK6, MEK1, and MEKK1 as well as Flag-tagged JNK1 constructs were generous gifts from Dr. C. Widmann (Department of Morphology and Cell Biology, University of Lausanne). GFP-ERK1 was obtained from Addgene. Plasminds encoding HA-MLK3 and HA-TAK1 were generous gifts form Dr. L. B. Holzman (University of Michigan Medical School) and Dr. J. Ninomiya-Tsuji (North Carolina State University), respectively.

The G*ATA4* Luciferase Reporter (Vector 0.7-kb ANF- luciferase plasmid) was provided by Mona Nemer (Montreal, Canada).

Vectors encoding Flag-AKAP-Lbc S1565A, AKAP-Lbc S1565A-GFP, Flag-tagged AKAP-Lbc fragments encoding residues 1-503, 504-1000, 1001-1387, 1388-1922, 1923-2336, 2337-2817, as well as 14-3-3β -GFP were described previously [42].

Expression and purification of recombinant proteins in bacteria

GST fusion proteins of AKAP-Lbc, p38α, MKK3 and MLTK were expressed using the bacterial expression vector pGEX-4T1 in the BL21DE3 strain of *Escherichia coli* and purified. Exponentially growing bacterial cultures were incubated 16 hours at 16°C with 1mM IPTG, and subsequently subjected to centrifugation. Pelleted bacteria were lysed in buffer D (20mM Tris pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 1% (w/v) Triton-X-100, 1µg/ml aprotinin, 2µg/ml leupeptin, 2µg/ml pepstatin, 0,1mM PMSF), sonicated and centrifuged at 38,000 x g for 30 min at 4 ˚C. After incubating the supernatants with glutathione Sepharose beads (Pharmacia) for 2 h at 4 ˚C, the resin was washed five times with 10 bed volumes of buffer D and stored at 4 ˚C.

His_6-tagged fusion proteins of PKNα and AKAP-Lbc were expressed using the bacterial expression vector pET30 in BL21DE3 bacteria and purified. Bacterial extracts containing His_6-tagged fusion proteins were prepared in buffer E (20mM Hepes pH 7.8, 500mM NaCl, 10mM imidazole, 1mM benzamidine, 2µg/ml leupeptin, 2µg/ml pepstatin). After a 1 min sonication, the lysates were centrifuged at 38,000 x g for 30 min at 4 ˚C. The His_6-tagged fusion proteins were purified by incubating the supernatant with Nickel-NTA chelating resin (Amersham Pharmacia Biotech) for 1 h at 4 ˚C. The resin was then washed 5 times with 10 bed volumes of buffer E and stored at 4 ˚C. His_6-tagged fusion proteins were eluted from the resin with 20mM Hepes pH 7.8, 500mM NaCl, 300 mM imidazole, 1mM benzamidine, 2µg/ml leupeptin, 2µg/ml pepstatin for 1h at room temperature, dialyzed and stored at –20 ˚C. The protein content of the eluates was assessed by Coumassie staining of SDS-PAGE gels.

Cell culture and transfections

HEK-293 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and gentamycin (100 μg/ml) and transfected at 50-60% confluency in 100 mm dishes using or the calcium-phosphate method. For the

overexpression of constructs containing the full-length AKAP-Lbc, HEK-293 cells were transfected at 80% confluency in 100 mm or 35 mm dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, cells were grown for 48 h in DMEM supplemented with 10% fetal calf serum before harvesting. The total amount of transfected DNA was of 10-24 μg /100 mm dish and 1-4μg /35mm dish.

Rat neonatal cardiomyocyte preparation

Rat neonatal ventricular myocytes were prepared from 1- to 2-days-old Sprague-Dawley rats. Excised hearts were digested by three cycles of enzymatic digestion at 37ºC for 15 min, using a mixture of 0.45 mg/ml collagenase type II (Worthington) and 1mg/ml pancreatine (Sigma), followed by centrifugation (800 rpm, 10min). The cells contained in the final pellet were suspended in dark medium (DMEM:M199 (4:1) medium supplemented with 1% penicillin/streptomycin, 1% hepes, 5% fetal calf serum and 10% horse serum (Invitrogen, Gibco)), and seeded on T75 cell culture flasks to deplete fibroblast. After two sequential steps of 1h of differential plating, non adherent neonatal myocytes were seeded in cell culture dishes pre-coated with 1% gelatine. After 24h the medium was changed and cells were cultured in dark medium. Cardiomyocyte culture purity was >95% as assessed by immunocytochemistry using an anti-actinin monoclonal antibody.

Production of lentiviruses

VSV-G pseudotyped lentiviruses were produced by cotransfecting 293-T cells with 20 µg of pAB286.1 or pSD28 vectors [205], pAB286.1-AKAP-Lbc shRNA vectors or pSD28-AKAP-Lbc shRNA, pAB286.1-AKAP-Lbc shRNA mutated or pSD28-AKAP-Lbc shRNA mutated [198], 15 µg of pCMVDR8.91 and 5 µg of pMD2.VSVG [204] using the calcium phosphate method. Culture medium was replaced by serum free DMEM at 12 hours after transfection. Cell supernatants were collected 48h later, filtered through a 0.45 mm filter unit, concentrated using Centricon-Plus-70 MW 100,000 columns (Millipore). Virus titers were determined by infecting 293-T cells using serial dilutions of the viral stocks and by scoring the number of puromycin-resistant clones at 6 days after infection (for pAB vectors) or scoring the number of GFP positive cells at 6 days after infection (for pSD28 vectors). Titers determined using these method were between $3x10^8$ and $8x10^8$ transducing units

(TU)/ml for viruses generated from pAB286.1 vectors and between $2x10^8$ and $5x10^8$ TU/ml for viruses generated from pSD28 vectors.

Lentiviral infection of HEK-293 cells

HEK-293 cells were infected at 60% confluency using pAB286.1-based lentiviruses encoding wild type or mutated AKAP-Lbc shRNAs at a multiplicity of infection (MOI) of 20 in the presence of 8μg/ml of polybrene. Two days after infection puromycin was added to the culture medium at a final concentration of 2 μg/ml. After four days of selection puromycin resistant cells were collected and amplified in selective medium containing puromycin at a final concentration of 2 μg/ml.

Lentiviral infection of rat neonatal cardiomyocytes

Rat neonatal cardiomyocytes were infected at 90% confluency using pSD28 based lentiviruses encoding wild type or mutated AKAP-Lbc shRNAs at a multiplicity of infection (MOI) of 50 in the presence of 8μg/ml of polybrene. 24h late ththe medium has been changed with the same volume of Maintainig medium. 48 hour later the cardiomyocyte have been subjected to serum starvation for 24 h with Light medium.

Real-Time PCR

Determination of the mRNAs levels of ANF, and skeletal α-actin in rat neonatal cardiomyocytes was carried out by real-time RT-PCR analysis by using a LightCycler Instrument (Roche Applied Science). Total mRNA was extracted from rat cardiomyocytes, and single-strand cDNA was synthesized from 2.5 μg of total RNA by using random hexamers (Applied Biosystems) and SuperScript II reverse transcriptase (Invitrogen). RT-PCR reactions were prepared by using a LightCycler kit (Eurogentec, Belgium) in a final volume of 20 μl containing 125 ng of reverse-transcribed total RNA and 0.5 μl of SYBR Green in the presence of the following forward and reverse primers described. Real-time PCR reactions were performed using the following forward (F) and reverse (R) primers: rat ANF (F) 5'-ATTTCAAGAACCTGCT-3' and (R) 5'-TCCTGTCAATCCTACC-3'; rat skeletal α-actin (F) 5'-TCTTGTGTGTGACAAC-3' and (R) 5'-

ATTTGAGTCATCTTCTCC-3', Glyceraldehyde3-phosphate dehydrogenase (F) 5'-GTCGGTGTGAACGGATTTGG-3' and (R) 5'-ACTCCACGACATACTCAGC-3'. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as invariant internal control. The relative amount of all mRNAs was calculated by using the LightCycler analysis software Version 3.5.

Dual Luciferase assay

RNVMs infected have been transfected with G*ATA4 Luciferase* Reporter and with pcmv-Renilla in a ratio of 7:1 with lipofectamine. 24 hours after trasfection cells were starved with Light medium for 24h. Following the starvation cardiomyocyte were stimulated for 24h with or without phenylephrine (PE) 10^{-4} M. Cells were washed 2 times with PBS cold and subsequently lysed (active lysis) with lysis buffer provide by promega kit (Dual-Glo® Luciferase Assay System). Lysates were store at -20°C overnight. The day after all samples were centrifuged at 10000 rpm for 1 minute at 4°C and then 20 µl of each samples were loaded in a reading plate. Dual luciferase values were read by the luminometer mithras LB 940 (Berthold).

In vitro GST pulldown experiments

For *in vitro* GST pulldowns, 100nM of bacterially purified His_6-tagged fragments encompassing PKNα residues 1-305 and 305-942 as well as AKAP-Lbc residues 1388-1922 were incubated with glutathione-sepharose beads (Amersham Biosciences) coupled to GST, or to GST-fusion proteins of p38α, MKK3, MLTK, or of the AKAP-Lbc fragment encompassing residues 1388-1922 in 0.5 ml of buffer A (20 mM Tris pH 7.4, 150 mM NaCl, 1% (w/v) Triton-X-100, 5 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF) for 4h at 4˚C. The beads were then washed five times with buffer A containing 300mM NaCl, resuspended in SDS-PAGE sample buffer (65 mM Tris, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, pH 6.8) and boiled for 3 min at 95°C. Eluted proteins were analyzed by SDS-PAGE and Western blotting.

Immunoprecipitation Experiments (cardiomyocytes)

For immunoprecipitation experiments, 7 *10^6 RNVMs grown in 100-mm dishes in 10 ml of DARK medium. 48 hours later RNVMs were lysed in 1 ml of buffer 1 (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% (wt/vol) Triton X-100, 0,1% Na deoxycholate, 5mg/ml aprotinin/10 mg/ml leupeptin/1 mM PMSF). Cell lysates were incubated 4 h at 4°C on a rotating wheel. The solubilized material was centrifuged at 100,000 ´*g* for 30 min at 4°C, and the supernatants were dialyzed in 5 liters of buffer 2 (20 mM HEPES, pH 7.4, 150 mM NaCl, 0,5 % (wt/vol)) 2 hours at 4°C. Next the supernatants were dialyzed again in 5 liters of fresh buffer 2 overnights at 4°C. The day after the supernatants were incubated 2h at 4°C with 4µg of polyclonal anti-AKAP-Lbc or with 4µg of rabbit IgG (negative control) on a rotating wheel. Next all the samples were incubated with 20 µl of protein-A sepharose beads for 2 hours at 4°C on a rotating wheel. Following a brief centrifugation on a bench-top centrifuge, the pelleted beads were washed five times with buffer B and twice with PBS, and proteins were eluted in SDS/PAGE sample buffer (65 mM Tris/2% SDS/5% glycerol/5% 2-mercaptoethanol, pH 6.8) by boiling samples for 3 min at 95°C. Eluted proteins were analyzed by SDS/PAGE and Western blotting

Immunoprecipitation experiments (HEK 293 cells)

For immunoprecipitation experiments, HEK-293 cells grown in 100 mm dishes and expressing various constructs were lysed in 1 ml of buffer A. Cell lysates were incubated 1h at 4˚C on a rotating wheel. The solubilized material was centrifuged at 100,000 x g for 30 min at 4˚C and the supernatants were incubated 4h at 4˚C with 20µl of anti-FlagM2 affinity resin (Sigma) to immunoprecipitate overexpressed Flag-tagged proteins. Following a brief centrifugation on a bench-top centrifuge, the pelleted beads were washed five times with buffer C, twice with PBS and proteins eluted in SDS-PAGE sample buffer by boiling samples for 3 min at 95°C. Eluted proteins were analysed by SDS-PAGE and Western blotting. For immunoprecipitation of endogenous AKAP-Lbc complexes, HEK-293 cells were lysed in 1 ml of buffer B (20mM Hepes pH 7.4, 150mM NaCl, 1% Triton-X-100 and 1 mM PMSF). Soluble proteins were isolated by centrifugation as indicated above and incubated with or without 0.25mM dithiobis [succinimidyl propionate] for 1h at 4˚C. Crosslinking reactions were blocked by adding Tris pH7.4 to the lysate to a final

concentration of 50mM. Immunoprecipitations were performed as indicated previously [42] by incubating 3mg of lysate with 4µg of affinity-purified rabbit polyclonal anti-AKAP-Lbc antibodies (Covance).

p38α, JNK and MLTK activity assays.

Transfected HEK-293 cells grown in 100 mm dishes were lysed in 1 ml of buffer C (20 mM Tris pH 7.4, 150 mM NaCl, 1% (w/v) Triton-X-100, 10mM NaF, 10mM Na-pyrophosphate, 1mM Na-orthovanadate, 1mM glycerophophate, 5 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF). Cell lysates were incubated 10 min at 4˚C on a rotating wheel. The solubilized material was centrifuged at 100,000 x g for 30 min at 4˚C. 200µl of supernatant were incubated either with 2µl of mouse monoclonal anti-p38α antibodies (Cell Signaling technology) and 20µl of protein A-sepharose beads (Amersham) for 2h at 4˚C to immunoprecipitate endogenous p38α or with 20µl of anti-FlagM2 affinity resin (Sigma) for 1h at 4˚C to immunoprecipitate overexpressed Flag-tagged JNK1 or MLTK. Following a centrifugation on a bench-top centrifuge, the pelleted beads were washed three times with buffer C and twice with a buffer containing 50mM Tris pH 7.4 and 5mM $MgCl_2$. Immunoprecipitates containing p38α or JNK1 were incubated with 1µg of purified GST-ATF2 (Cell Signaling Technology) whereas those containing Flag-tagged MLTK were incubated with 1µg of purified GST-MKK3. Reactions were carried out in 50mM Tris pH 7.4, 5mM $MgCl_2$ and 1mM ATP-Na_2 for 30 min at 30˚C and ended by the addition of SDS-PAGE sample buffer and loaded on SDS-PAGE gels.

SDS-PAGE and Western blotting

Samples denatured in SDS-PAGE sample buffer were separated on acrylamide gels and electroblotted onto nitrocellulose membranes. The blots were incubated with primary antibodies and horseradish-conjugated secondary antibodies (Amersham) as previously indicated [189]. The following affinity purified primary antibodies were used for immunoblotting: affinity purified rabbit polyclonal anti-AKAP-Lbc (Covance, 0.1 mg/ml, 1:1000 dilution), mouse monoclonal anti-Flag (Sigma, 4.9 mg/ml, 1:2000 dilution), mouse monoclonal anti-GFP (Roche, 400µg/ml, 1:500 dilution), rabbit polyclonal anti-GFP (Roche 400µg/ml, 1:1000 dilution), rabbit polyclonal anti-HA (Sigma, 1:1000 dilution),

mouse monoclonal anti-HA (Sigma, 1:5000 dilution), rabbit polyclonal anti-phospho p38α (threonine 180 and tyrosine 182) (Cell Signaling Technologies, 1:1000 dilution), rabbit polyclonal and mouse monoclonal anti-p38α (Cell Signaling Technologies, 1:1000 dilution), rabbit polyclonal anti-phospho ATF2 (Threonine 71) (Cell Signaling Technologies, 1:1000 dilution), rabbit polyclonal anti-ATF2 (Cell Signaling Technologies, 1:1000 dilution), mouse monoclonal anti-phospho MKK3 (Serine 189 and Threonine 193) (Cell Signaling Technology, 1:500 dilution), mouse monoclonal anti-MKK3 (Assay designs, 1:1000 dilution), mouse monoclonal anti-MLTK (Abnova, 1:500 dilution), rabbit polyclonal anti-MLK3 (Cell Signaling Technology, 1:500 dilution), mouse monoclonal anti-PKNα (BD Biosciences Pharmingen, 1:1000 dilution), rabbit polyclonal anti-ERK1/2 (Santa Cruz Biotechnology, 1:500 dilution), rabbit polyclonal anti-phospho ERK1/2 (threonine 202 and tyrosine 204) (Santa Cruz Biotechnology, 1:500 dilution), rabbit polyclonal anti-JNK (Cell Signaling Technologies, 1:500 dilution), rabbit polyclonal anti-phospho JNK (threonine 183 and tyrosine 185) (Cell Signaling Technologies, 1:500 dilution), rabbit polyclonal anti-phospho Hsp27 (serine 82) (Cell Signaling Technologies, 1:500 dilution), rabbit polyclonal anti-Hsp27 (Cell Signaling Technologies, 1:500 dilution), rabbit polyclonal anti-phospho GATA4 (serine 105) (Sigma, 1:1000 dilution), rabbit polyclonal anti-GATA4 (Santa Cruz Biotechnology, 1:250 dilution), mouse monoclonal anti-actin (Sigma, 1:1000 dilution), mouse monoclonal anti-Histidine tag (Qiagen 100μg/ml, 1:1000 dilution).

Fluorescence Microscopy

RNVMs were grown for 24 h in the presence of maintaining medium. Cells were then incubated for 1 h with or without 10^{-4} M phenylephrine, washed twice with PBS, and then fixed for 10 min in PBS/3.7% formaldehyde, permeabilized for 5 min with 0.2% (wt/vol) Triton X-100 in PBS, and blocked for 1 h in PBS/1% BSA. The expression of the Hsp27 was assessed by incubating cardiomyocytes for 12 h with a 1:250 dilution of the rabbit anti-Hsp27 polyclonal antibody (Santa Cruz) followed by 1 h incubation with Rhodamine-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch). The expression of α-actinin was assessed by incubating cells for 1 h with a 1:500 dilution of mouse monoclonal antibodies against anti-α-actinin (Sigma) followed by 1 h incubation with rhodamine-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch). The

expression of actin was assessed by incubating cells for 1 h with a 1:1000 dilution of rhodamine-conjugated phalloidin (Jackson ImmunoResearch). The cells were mounted using Prolong (Molecular Probes).

RNVMs were transfected with pEGFP plasmid or with AKAP-Lbc 1585-1715 in pEGFP with lipofectamine. After 48 hours cells were starved for 24h and then stimulated for 24 h with or without 10^{-4} M phenylephrine. Cells were washed twice with PBS, and then fixed for 10 min in PBS/3.7% formaldehyde, permeabilized for 5 min with 0.2% (wt/vol) Triton X-100 in PBS, and blocked for 1 h in PBS/1% BSA. The expression of α-actinin was assessed by incubating cells for 1 h with a 1:500 dilution of mouse monoclonal antibodies against anti-α-actinin (Sigma) followed by 1 h incubation with rhodamine-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch). The cells were mounted using Prolong (Molecular Probes). Intrinsic GFP fluorescence, as well as immunofluorescent staining, was visualized using a Zeiss Axiophot fluorescence microscope.

Statistical analysis

Statistical significance was analyzed using a Kruskal-Wallis test followed by Mann-Whitney U tests with the Bonferroni corrections.

IV. RESULTS I: Characterization of the signaling pathway activated by the AKAP-Lbc signaling complex

These results have been published: ***Cariolato L, Cavin S, Diviani D. (2011.). A-kinase anchoring protein (AKAP)-Lbc anchors a PKN-based signaling complex involved in α1-adrenergic receptor-induced p38 activation. J. Biol. Chem. 286(10):7925-37 (see annexe)***

4.1 AKAP-Lbc activates p38 MAPK in HEK-293 cells.

While our previous findings indicate that AKAP-Lbc/RhoA complex is a key mediator of the growth responses induced by GPCRs including α1-ARs and AT1-Rs, the signaling pathways linking AKAP-Lbc to the activation of hypertrophic genes remain to be elucidated.

In this context, evidence collected over the past few years suggests that activated RhoA can regulate the transcription of genes involved in cardiac hypertrophy through the activation of transduction cascades involving protein kinases of the MAPK family including ERK, JNK and p38 [34, 141, 211]. Based on these findings we have raised the hypothesis that, in cardiomyocytes, AKAP-Lbc might induce hypertrophic gene expression by promoting the activation of MAPKs.

To initially address the hypothesis that AKAP-Lbc can activate members of the MAPK family, HEK-293 cells were transfected with an empty Flag vector or with the cDNA encoding a Flag-tagged mutant of AKAP-Lbc (AKAP-Lbc S1565A) displaying constitutive Rho-GEF activity and serum starved for 24 h. Cell lysates were prepared and the presence of active phosphorylated forms of ERK1/2, JNK and p38 determined by western blot using anti-phospho ERK1/2, anti-phospho JNK and anti-phospho p38 specific antibodies, respectively. Interestingly, we found that the active mutant of AKAP-Lbc could enhance p38 phosphorylation without affecting ERK phosphorylation and JNK phosphorylation (Figure 1A, 1B and 1C upper panel, lane 2). This suggests that the AKAP-Lbc signaling complex selectively activates p38.

4.2. RhoA mediates α1b-AR-mediated p38 MAPK activation.

Since AKAP-Lbc is a guanine nucleotide exchange factor that selectively activates RhoA, and since RhoA has been described as being an upstream activator of p38 MAPK in

cardiomyocytes, we determined whether the activity of this small molecular weight G protein might mediate the activation of p38 MAPK activity induced the α1b-AR.

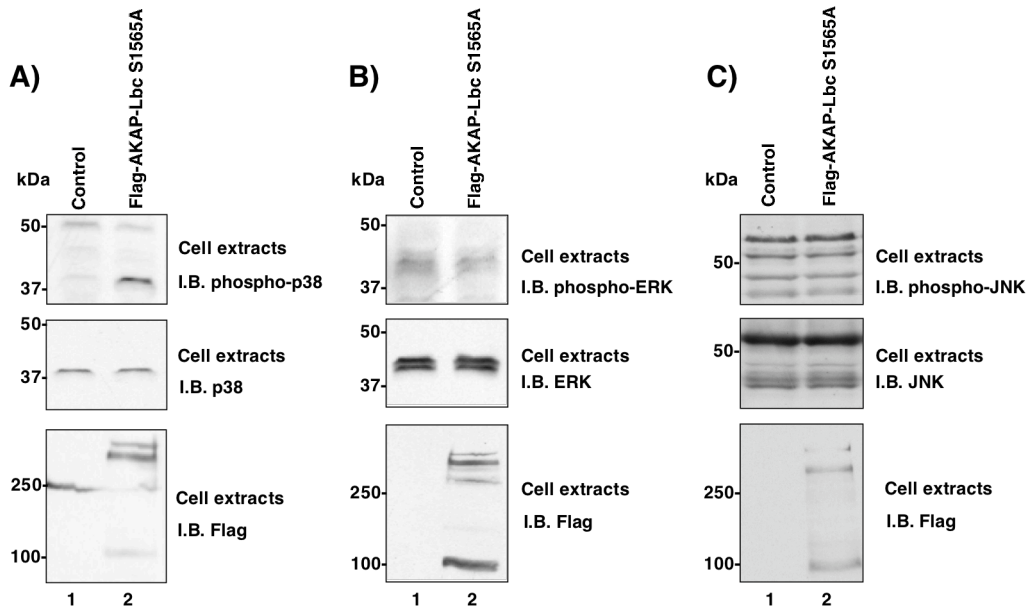


Figure 1: AKAP-Lbc activates p38 inside cells. A-C) HEK-293 cells expressing empty Flag vector (lane 1) or the Flag-tagged form of the constitutively active S1565A mutant of AKAP-Lbc (lane 2) were serum starved for 24 h. Proteins in the cell lysates were separated by SDS-PAGE and the phosphorylated form of p38, ERK1/2 and JNK were detected with phospho specific antibody of p38, ERK1/2 and JNK (upper panel). The amounts of total p38, ERK1/2 and JNK in the cell lysates were assessed using specific antibodies against p38, ERK1/2 and JNK (middle panel). The amounts of total Flag-tagged AKAP-Lbc in the cell lysates was assessed using monoclonal antibodies against the Flag epitope (lower panel).

To initially determine the implication of RhoA in the pathways linking α1b-ARs to the activation of ERK1/2, JNK and p38, HEK-293 cells expressing the HA-tagged α1b-AR were treated for 2h in the absence or presence of 1μg/ml of a cell permeable form of the C3 botulimun toxin and subsequently incubated with or without 10^{-4} M epinephrine for 10 min. Activation of ERK1/2, JNK in cell lysates was assessed by western blot using antibodies recognizing the phosphorylated forms of ERK1/2 and JNK. On the other hand, p38α activity was determined using a kinase assay that measured the ability of immunoprecipitated endogenous p38α to induce the phosphorylation of purified GST-ATF2. Interestingly, inhibition of RhoA impaired by 58% and 69% the ability of α1b-ARs to induce p38α activation under basal conditions and following epinephrine stimulation, respectively (Fig. 2A, panel 1, lanes 7 and 8;), without affecting α1b-AR-mediated phosphorylation of ERK1/2 and JNK (Fig. 3A, panel 1, lanes 7 and 8; Fig. 3B, panel 1 lanes 7 and8). This suggests that RhoA is involved in the pathway that links α1b-ARs to the activation of p38α.

To directly determine whether AKAP-Lbc can enhance p38α activation through its ability to activate RhoA, we assessed whether RhoA inhibition could affect the p38α activating

potential of the S1565A mutant of AKAP-Lbc, which displays constitutive Rho-GEF activity. HEK-293 cells transfected with the Flag-tagged AKAP-Lbc S1565A mutant were serum starved for 24h and incubated for 2h in the absence or presence of 1µg/ml of C3 botulinum toxin. As shown in Fig. 2B, overexpression of the Flag-tagged AKAP-Lbc S1565A mutant induced a 2.9 fold enhancement of p38α kinase activity, which was reduced after RhoA inhibition (Fig.2B, panel 1, lanes 2 and 3). Altogether, these findings indicate that the AKAP-Lbc/RhoA complex specifically mediates α1-AR-induced p38α activation.

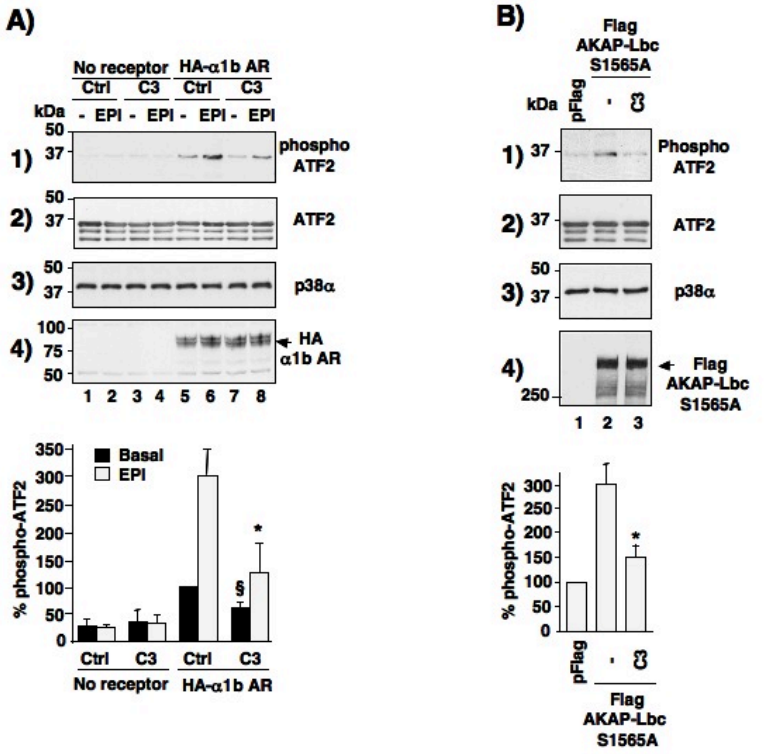


Figure 2: AKAP-Lbc mediates α1b-AR-induced p38a activation. A) HEK-293 cells were transfected with the empty pRK5 plasmid or the cDNA encoding the HA-tagged α1b-AR. After a 24 h serum starvation, cells were incubated for 2 h with or without 1µg/ml of purified C3 toxin and incubated for 10 min with or without (Ctrl) 10^{-4} M epinephrine (EPI). Cell lysates where subjected to immunoprecipitation using anti-p38α monoclonal antibodies. Kinase reactions were performed by incubating p38α immunoprecipitates with 1 µg of purified GST-ATF2 and in the presence of ATP. Phospho-GST-ATF2 was detected by immunoblot using rabbit polyclonal antibodies recognizing phospho-threonine 71 of ATF2 (panel 1). The amounts of GST-ATF2, p38α and HA-α1b-ARs were assessed using polyclonal antibodies against ATF2 (panel 2), p38α (panel 3) and the HA epitope (panel 4), respectively. Quantitative analysis of phosphorylated ATF2 was obtained by densitometry. The amount of phospho-ATF2 was normalized to the total amount of ATF2 and p38α. Results are expressed as mean ± S.E. of 3 different experiments. § $p < 0.05$ as compared with phospho-ATF2 levels measured in untreated control cells expressing HA-α1b-ARs. * $p < 0.05$ as compared with phospho-ATF2 levels measured in epinephrine-treated control cells expressing HA-α1b-ARs. **B)** HEK-293 cells were transfected with cDNA encoding the Flag-tagged S1565A mutant of AKAP-Lbc. After a 24 h serum starvation, cells were incubated for 2 h with or without 1µg/ml of purified C3 toxin. Kinase activity of immunoprecipitated p38α and detection of phospho-ATF2 (panel 1) and ATF2 (panel 2) and p38α (panel 3) in cell lysates was determined as indicated in A). Expression of the Flag-tagged AKAP-Lbc S1565A mutant was assessed using monoclonal antibodies against the Flag-tag (panel 4). Quantitative analysis of phosphorylated ATF2 was obtained by densitometry as indicated before. * $p < 0.05$ as compared with phospho-ATF2 levels measured in untreated cells expressing Flag-AKAP-Lbc S1565A.

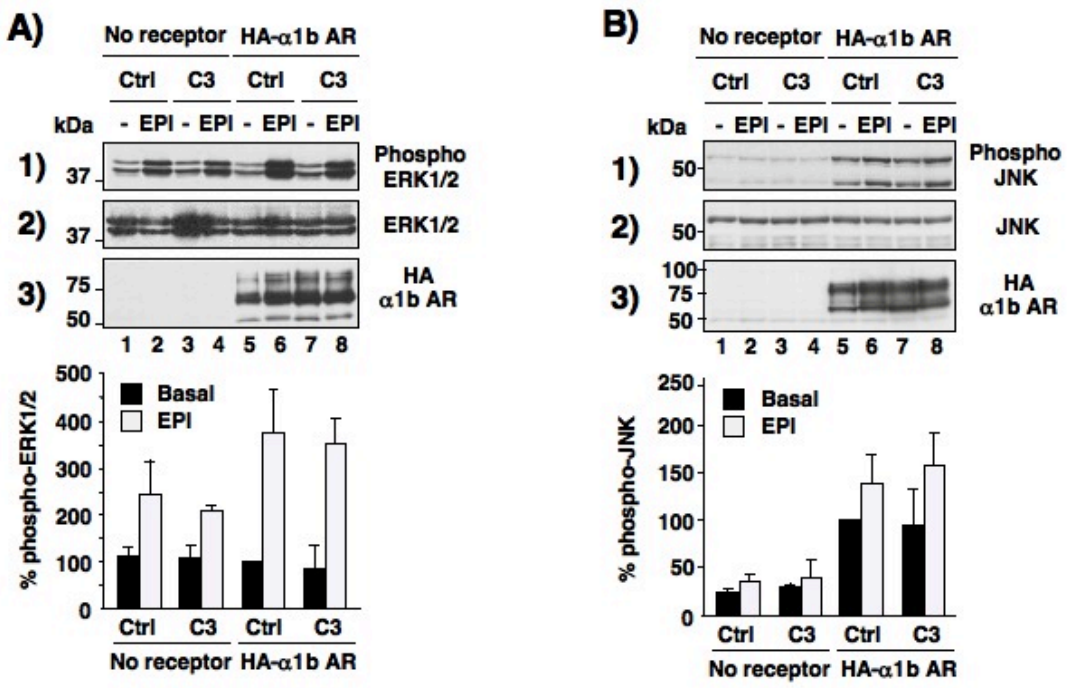


Figure 3 RhoA does not mediate α1b-AR-induced activation of ERK1/2 and JNK. A, B) HEK-293 cells were transfected with the plasmid encoding the HA-tagged α1b-AR. After a 24 h serum starvation, cells were incubated for 2 h with or without 1μg/ml of purified C3 toxin and subsequently stimulated for 10 min with or without (Ctrl) 10^{-4} M epinephrine (EPI). Phosphorylated ERK1/2 and JNK were detected by immunoblot using rabbit polyclonal antibodies recognizing phosphorylated threonine 202 and phosphorylated tyrosine 204 of ERK and phosphorylated threonine 183 and phosphorylated tyrosine 185 of JNK1, respectively (A, B, panel 1). The amounts of total ERK1/2, JNK and HA-α1b-ARs in the cell lysates were assessed using antibodies against ERK1/2 (A, panel 2), JNK (B, panel 2) and the HA epitope (A, B, panel 3), respectively. Results are expressed as mean ± S.E. of 4 different experiments. Quantitative analysis of phosphorylated ERK1/2 and JNK was obtained by densitometry. The amount of phosphorylated ERK1/2 and JNK was normalized to the total amount of ERK1/2 and JNK. Results are expressed as mean ± S.E. of 3 different experiments.

4.3 AKAP-Lbc is involved in α1b-AR-mediated p38 MAPK activation

Our previous results indicate that AKAP-Lbc can be activated downstream of α1b-ARs in primary cultures of rat neonatal cardiomyocytes. Based on these findings and on our current results suggesting that AKAP-Lbc can activate p38, we initially investigated the possibility that AKAP-Lbc could mediate the activation of p38 downstream of α1b-ARs. To address this hypothesis, we decided to determine the impact of AKAP-Lbc silencing in HEK-293 cells on the ability of α1b-ARs to induce the activation of p38.

AKAP-Lbc silencing was achieved by infecting cells using lentiviruses encoding two distinct short hairpin (sh) RNAs directed against a sequence within the DH domain (shRNA1) and N-terminal regulatory region (shRNA2) of AKAP-Lbc, respectively. Both

shRNAs could inhibit AKAP-Lbc expression by about 90% as compared to cells infected with control lentiviruses (Fig.4A, panel 5).

Infected cells were transfected with the cDNA encoding the HA-tagged α1b-AR, serum starved for 24 h and incubated in the absence or presence of epinephrine for 10 min. p38α was then immunoprecipitated and its activity assessed as indicated above.

Silencing of AKAP-Lbc expression significantly reduced the ability of α1b-ARs to induce p38α activation both under basal conditions and following epinephrine stimulation. Basal p38α activation was inhibited between 67% (shRNA1) and 74% (shRNA2) whereas inhibition of epinephrine-induced p38α activation was between 63% (shRNA1) and 72% (shRNA2) (Fig. 4A, panel 1, lanes 9-12). On the other hand, control experiments revealed that the ability of α1b-ARs to promote phosphorylation of endogenous ERK1/2 and JNK was not affected by AKAP-Lbc silencing (Fig. 4B, panel 1 and 3, lanes 7 and 8).

Interestingly, re-expression of a silencing-resistant mutant of AKAP-Lbc (2) in silenced cells rescued the ability of α1b-AR to promote the activation of p38α (Fig. 5, panel 1, lanes 5 and 6).

These results strongly suggest that AKAP-Lbc specifically contributes to the activation of p38α MAPK induced by α1b-ARs and its inhibition was strictly dependent on reduced AKAP-Lbc expression and not due to an off-target effect.

4.4 AKAP-Lbc interacts with p38

Our current findings reveal that AKAP-Lbc and RhoA mediate the activation of p38 MAPK but not that of ERK1/2 and JNK downstream of α1b-ARs (Figure 2-3-4). This suggests the existence of molecular mechanisms that allow the AKAP-Lbc signaling complex to select and activate the p38 effector pathway. One attractive hypothesis would be that AKAP-Lbc and p38 might be maintained within the same macromolecular unit. In this configuration, activating signals could be integrated by AKAP-Lbc and rapidly transmitted to p38.

To assess whether AKAP-Lbc could form a complex with p38, we determined whether endogenous p38 could be co-immunoprecipitated with Flag-tagged AKAP-Lbc from lysates of transfected HEK-293 cells.

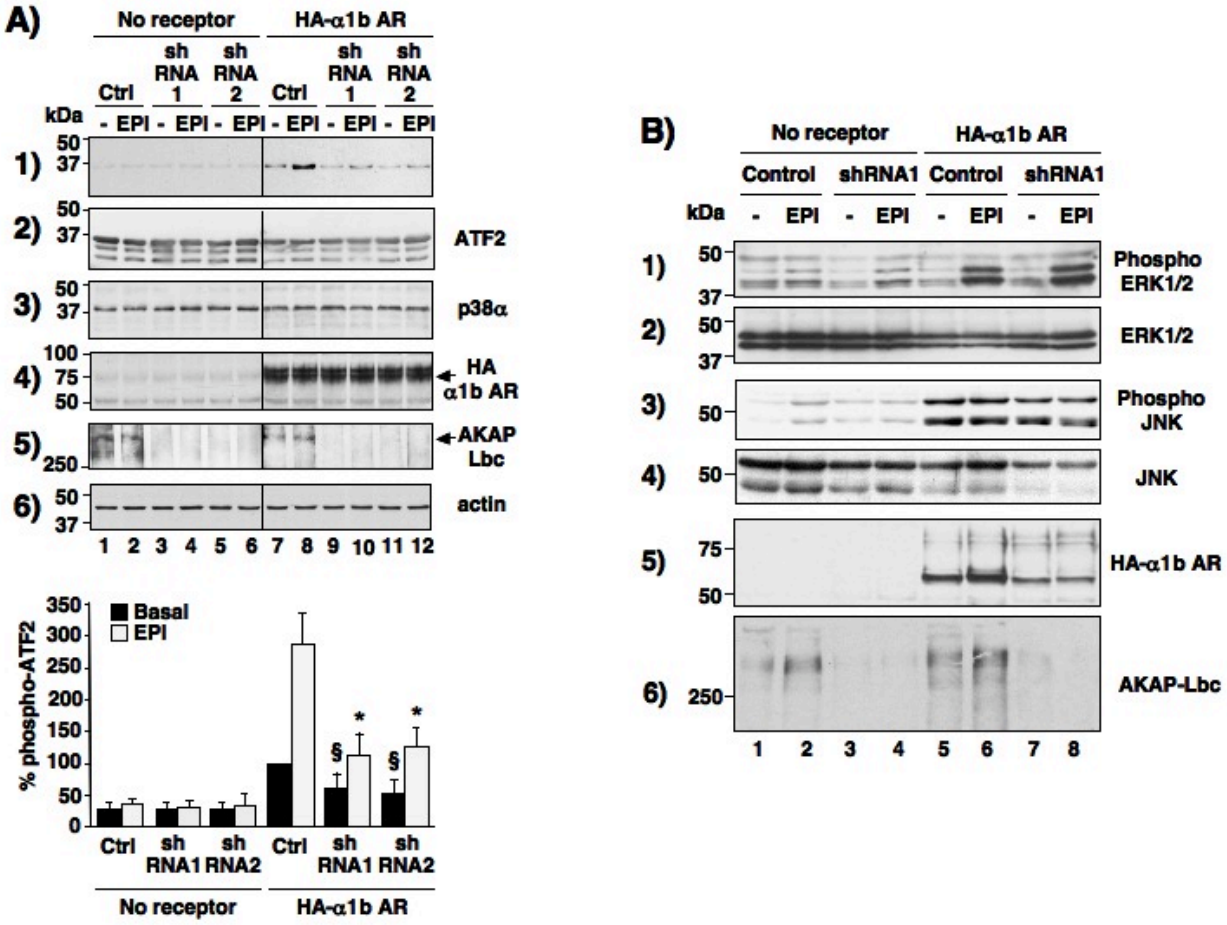


Figure 4 AKAP-Lbc is involved in α1b-AR-mediated p38α MAPK activation. A) HEK-293 cells infected with control lentiviruses or lentiviruses encoding AKAP-Lbc shRNAs were transfected with the empty pRK5 plasmid or the cDNA encoding the HA-tagged α1b-AR. After a 24 h serum starvation, cells were incubated for 15 min with or without (Ctrl) 10^{-4} M epinephrine (EPI), lysed and subjected to immunoprecipitation using monoclonal anti-p38a antibodies. Kinase reactions and detection of phospho-ATF2 (panel 1), ATF2 (panel 2), p38α (panel 3), and HA-α1b-ARs (panel 4) were performed as indicated in A). Expression of endogenous AKAP-Lbc and actin was detected using affinity purified anti-AKAP-Lbc polyclonal antibodies (panel 5) and anti-actin monoclonal antibodies (panel 6). Quantitative analysis of phosphorylated ATF2 was obtained by densitometry. The amount of phospho-ATF2 was normalized to the total amount of ATF2 and p38α. Results are expressed as mean ± S.E. of 3 different experiments. § $p< 0.05$ as compared with phospho-ATF2 levels measured in untreated control cells expressing HA-α1b-ARs. * $p< 0.05$ as compared with phospho-ATF2 levels measured in epinephrine-treated control cells expressing HA-α1b-ARs. **B)** HEK-293 cells infected with control lentiviruses or lentiviruses encoding AKAP-Lbc shRNAs directed against bases 6688-6706 of the human AKAP-Lbc sequence were subsequently transfected with the empty pRK5 vector (no receptor) or the vector encoding the HA-tagged a1b-AR. After a 24 h serum starvation, cells were incubated for 15 min with or without 10^{-4} M epinephrine (EPI) and lysed. Phosphorylation of ERK1/2 (panel1) and JNK (panel 3) was assessed by western blot using anti-phospho specific.The amounts of ERK1/2, JNK, HA-a1b-ARs and AKAP-Lbc in the cell lysates were assessed using polyclonal antibodies against ERK1/2 (panel 2) and JNK (panel 4), monoclonal antibodies against the HA epitope (panel 5), and affinity purified polyclonal antibodies against AKAP-Lbc (panel 6), respectively.

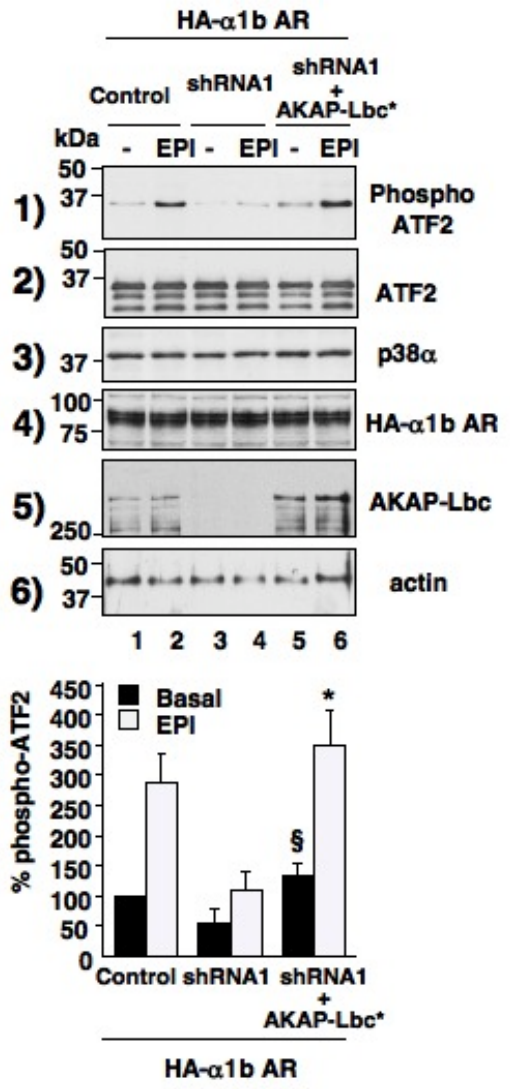


Figure 5: Rescue of shRNA-mediated inhibition of α1b-AR-induced p38α activation. HEK-293 cells infected with control lentiviruses or lentiviruses encoding shRNAs directed against bases 6688-6706 of the human AKAP-Lbc sequence (shRNA1) were subsequently transfected with the plasmid encoding the HA-tagged a1b-AR in the combination with the empty Flag vector (lanes 1-4) or with the cDNA encoding the Flag-tagged silencing resistant mutant of AKAP-Lbc (lanes 5 and 6). After a 24 h serum starvation, cells were incubated for 15 min with or without 10^{-4} M epinephrine (EPI). Cell lysates where subjected to immunoprecipitation using anti-p38a polyclonal antibodies. Kinase reactions were performed by incubating p38α immunoprecipitates with 1 μg of purified GST-ATF2 and in the presence of ATP. Phospho-GST-ATF2 was detected by immunoblot using rabbit polyclonal antibodies recognizing phospho-threonine 71 of ATF2 (panel 1). The amounts of GST-ATF2, p38α, HA-a1b-ARs, AKAP-Lbc and actin were assessed using antibodies against ATF2 (panel 2), p38a (panel 3) and the HA epitope (panel 4) and AKAP-Lbc (panel 5), respectively.
Quantitative analysis of phosphorylated ATF2 was obtained by densitometry. The amount of phospho-ATF2 was normalized to the total amount of ATF2 and p38α. Results are expressed as mean ± S.E. of 3 different experiments. § $p< 0.05$ as compared with phospho-p38 levels measured in untreated cells infected with lentiviruses encoding shRNA1. * $p< 0.05$ as compared with phospho-p38 levels measured in epinephrine-treated cells infected with lentiviruses encoding shRNA1.

As shown in Fig. 6A, anti-Flag antibodies could immunoprecipitate HA-p38α from cells expressing the Flag-tagged AKAP-Lbc, but not from cells transfected with the empty pFlag vector (Fig. 6A, upper panel, lanes 1 and 2). Using a similar approach we could show that HA-JNK1 and GFP-ERK1 do not form stable complexes with AKAP-Lbc (Fig. 6B and 6C, upper panel, lanes 1 and 2). These results suggest that AKAP-Lbc specifically binds and activates p38α. The p38 family of MAPK is constituted by four members (p38α, p38β, p38γ and p38δ)[212]. Co-immunoprecipitation experiments performed using the different recombinant p38 kinases indicate that Flag-AKAP-Lbc interacts mainly with p38α, to a lesser extent with p38β, but not with p38γ and p38δ (results not shown).

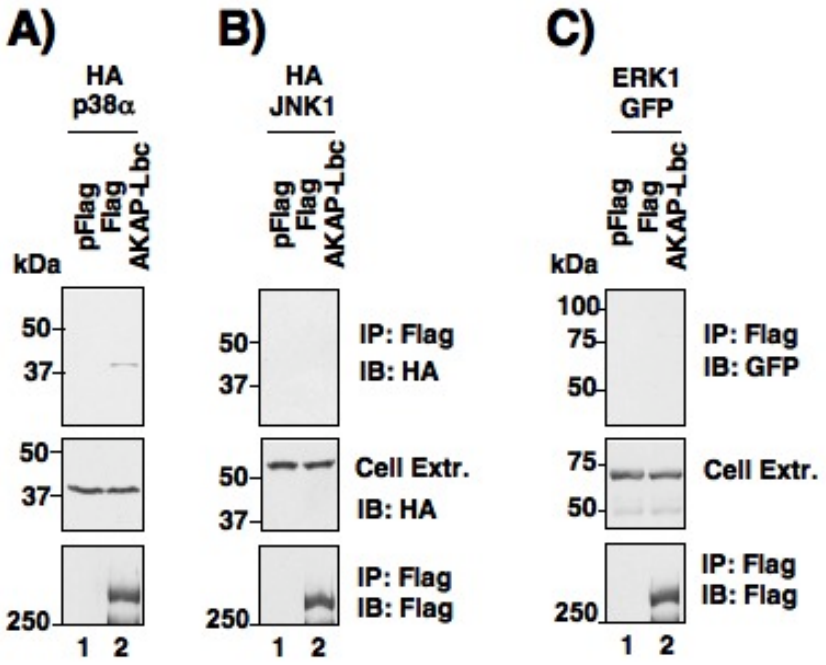


Figure 6: AKAP-Lbc interacts with p38α. A-C) Extracts from HEK-293 cells transfected with the plasmids encoding HA-tagged p38α (A), HA-tagged JNK1 (B) or GFP-tagged ERK1 (C) in combination with the empty Flag vector (lane 1) or the vector encoding Flag-tagged AKAP-Lbc (lane 2) were subjected to immunoprecipitation with anti-Flag antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using anti-HA monoclonal antibodies to detect HA-p38α and HA-JNK1 (A-B, upper and middle panels), anti-GFP polyclonal antibodies to detect GFP-ERK1 (C, upper and middle panels) or anti-Flag monoclonal antibodies to detect Flag-AKAP-Lbc (lower panels).

4.5 AKAP-Lbc assembles a p38 activation module

MAPKs are activated by a protein kinase cascades in which the MAPK is phosphorylated and activated by a MAP kinase kinase (MAPKK) that is, in turn, phosphorylated and activated by a MAP kinase kinase kinase (MAPKKK). As mentioned in the introduction it is well established that p38α can be activated by the MAPKKs MKK3, MKK4 and MKK6 [213]. In turn, these three p38-activating kinases can be phosphorylated and activated by several MAPKKKs such as TAK1, members of the mixed lineage kinase (MLK) family including MLK3, MLTK and DLK, and several members of the MEKK family of protein kinases [213, 214]. These MAPK cascades are organized into signaling complexes to create functional MAPK modules. Interestingly recent studies have shown that RhoA can promote the activation of p38 through a signaling pathway that includes PKN, MAPKKKs of the MLK family and MAPKKs such MKK3 and MKK6. Based on these observations we have investigated the possibility of whether these kinases known to act upstream of p38 would also associate with AKAP-Lbc.

To address this point, we performed coimmunoprecipitation experiments from HEK-293 cells that were transiently transfected with the cDNA encoding the HA-tagged forms of the MAPKKs MKK3, MKK4 and MKK6 (Fig. 7A) as well as of the MAPKKKs MLK3, MEK1, TAK1 (Fig. 7B) and MLTK (Fig. 7C) in combination with the empty Flag vector (pFlag) or the Flag-tagged AKAP-Lbc. After immunoprecipitating the anchoring protein using anti-Flag antibodies, anti-HA antibodies were used to immunoblot the immunoprecipitated samples. Western blots revealed that MKK3 (Fig.7A, upper panel, lane 5) as well as kinases belonging to MLK family including MLK3 and MLTK (Fig.7B, upper panel, lane 4; Fig. 7C upper panel, lane 2) could specifically co-immunoprecipitate with AKAP-Lbc whereas the MAPKKs MKK4 and MKK6 as well as the MAPKKKs MEKK1 and TAK1 did not (Fig. 7A upper panel, lanes 4 and 6, Fig. 7B, upper panel, lanes 5 and 6). These results suggest that AKAP-Lbc can interact with a signaling module composed of p38α, its upstream kinase MKK3, and MAPKKKs of the MLK family such as MLK3 or MLTK.

However, MLKs have never been reported as direct effectors of RhoA. Accordingly, control experiments performed in our laboratory using purified kinases failed to detect MLK activation by RhoA (results not shown). This raises the question of how the AKAP-Lbc/RhoA complex can transmit signals to the MLK-MKK3-p38α module. In this context, previous evidence indicates that protein kinase Nα (PKNα), a well-characterized effector of RhoA, can act as an upstream activating kinase of MLTK [215]. Based on these findings, we raised the hypothesis of whether PKNα could also bind to AKAP-Lbc. Interestingly, western blots performed on Flag-AKAP-Lbc immunoprecipitates indicate that endogenous PKNα can co-immunoprecipitate with the anchoring protein (Fig. 7D, upper panel, lane 2).

In a similar set of experiments, we could show that p38α, MKK3, MLTK and PKNα endogenously expressed in HEK293 cells could form a complex with endogenous AKAP-Lbc. This is shown by the fact that p38α, MKK3, MLTK and PKNα could be detected in AKAP-Lbc immunoprecipitates (Fig. 8A, upper and middle panels, lane 3; Fig. 8 B, upper and middle panels, lane 3). On the other hand, no interaction between endogenous AKAP-Lbc and MLK3 could be detected (Fig. 8C, upper panel, lane 3). Therefore, while overexpressed AKAP-Lbc has the potential of binding MLK3 and MLTK (Fig. 7B and 7C), endogenous AKAP-Lbc seems to preferentially assemble a complex that specifically contains MLTK. Collectively these results suggest that AKAP-Lbc can assemble a large signaling complex containing PKNα, MLTK, MKK3 and p38α that can link RhoA activation to the stimulation of the p38 transduction pathway.

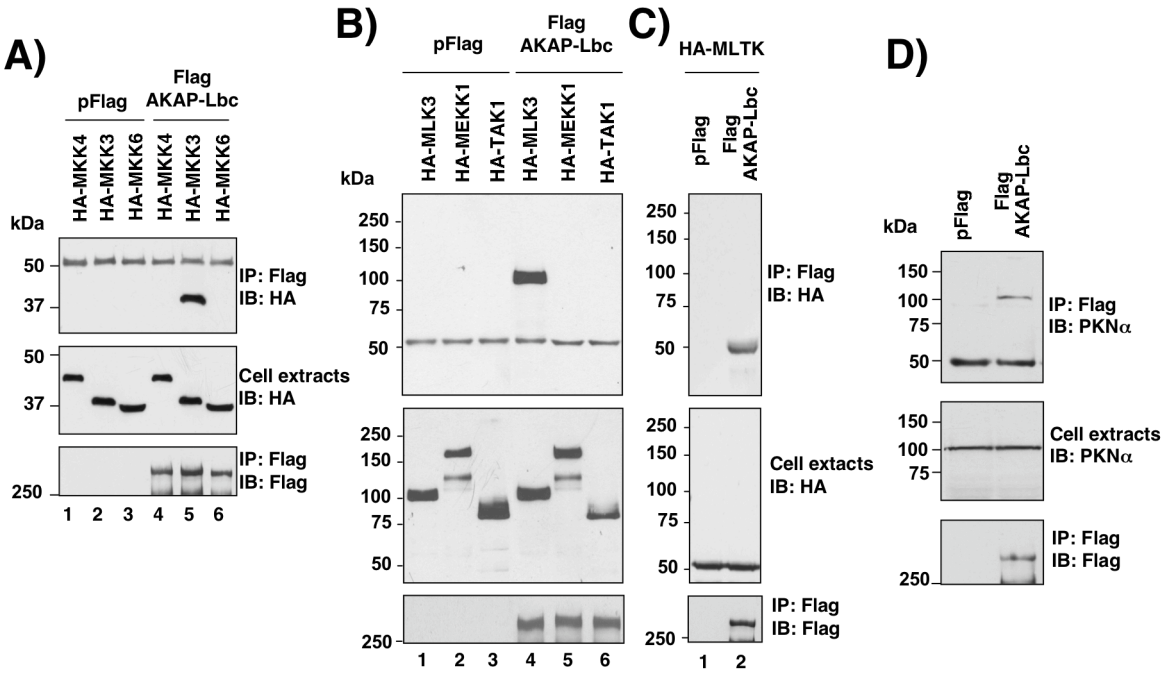


Figure 7: AKAP-Lbc assembles a p38α activation complex. A) HEK-293 cells were transfected with the plasmids encoding HA-tagged MKK4, MKK3, or MKK6 in combination with the vector encoding Flag-tagged AKAP-Lbc. Immunoprecipitations and western blots were performed as indicated in A). **B-C)** HEK-293 cells were transfected with the plasmids encoding HA-tagged MLK3, MEKK1, TAK1 (E) or MLTK (F), in combination with the empty Flag vector or the plasmid encoding Flag-tagged AKAP-Lbc. Immunoprecipitations and western blots were performed as indicated in A). **D)** Extracts from HEK-293 cells transfected with the empty Flag vector or the plasmid encoding Flag-tagged AKAP-Lbc were subjected to immunoprecipitation with anti-Flag antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using anti-PKNα monoclonal antibodies. Results are representative of at least three independent experiments.

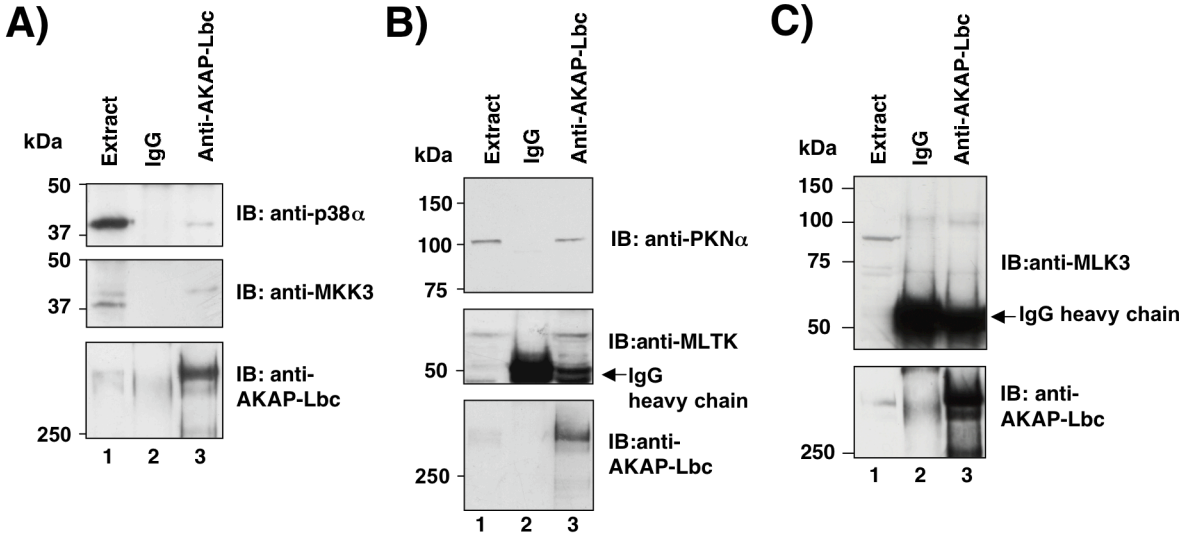


Figure 8. AKAP-Lbc interacts with endogenous p38α, MKK3, MLTK and PKNα. HEK-293 cell extracts were subjected to immunoprecipitation with either non-immune IgGs or affinity purified anti-AKAP-Lbc polyclonal antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using either anti-p38α (A, upper panel), anti-MKK3 (A, middle panel), anti-PKNα (B, upper panel) anti-MLTK (B, middle panel), anti-MLK3 (C, upper panel) or affinity purified anti-AKAP-Lbc polyclonal antibodies (lower panels). Results are representative of at least three independent experiments.

4.6 Mapping of the kinase binding sites on AKAP-Lbc.

To identify the binding site for p38α as well as its upstream activating kinases on AKAP-Lbc, we initially generated a series of Flag-tagged AKAP-Lbc fragments encompassing residues 1-503, 504-1000, 1001-1387, 1388-1922, 1923-2336 and 2337-2817 (Fig. 9A). The fragments were initially expressed in HEK-293 cells in combination with HA-MKK3 and interactions assessed by co-immunoprecipitation. The Flag-tagged fragments were immunoprecipitated from cell lysates using anti-Flag antibodies and the presence of associated kinases detected using anti-HA antibodies. Our results indicate that MKK3 interact exclusively with the fragment of AKAP-Lbc included between residues 1388 and 1922 (Fig. 9B, upper panel, lane5).

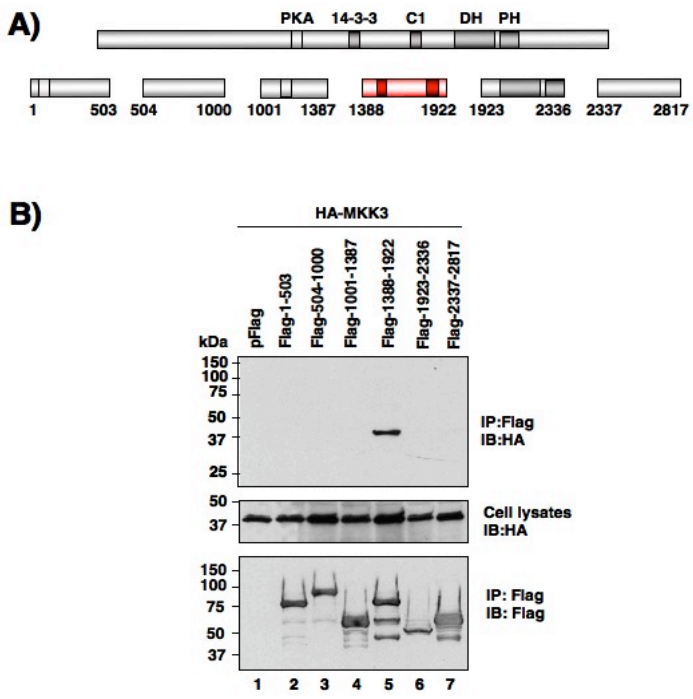


Figure 9: Initial mapping of the MKK3 interaction site on AKAP-Lbc. **A)** Schematic representation of the Flag tagged AKAP-Lbc fragments used for the mapping of the MKK3 binding site on AKAP-Lbc. PKA and 14-3-3 binding sites as well as C1, DH and PH domains are shown. For each fragment, the position of the first and the last amino acid is indicated. **B)** Extracts from HEK-293 cells expressing HA-MKK3 in combination with the empty Flag vector or the plasmids encoding the various Flag-tagged AKAP-Lbc fragments indicated in A) were subjected to immunoprecipitation with anti-Flag antibodies. Western blots of the immunoprecipitates and cell extracts were revealed using anti-HA monoclonal antibodies to detect HA-MKK3 (upper and middle panels) or monoclonal anti-Flag antibodies to detect the Flag-tagged fragments (lower panel).

We have subsequently further narrowed the binding site using shorter Flag-tagged AKAP-Lbc fragments derived from the region 1388-1922 (Fig. 10A). As shown in Fig. 10B, our results indicate that HA-MKK3 interact with AKAP-Lbc in a minimal region encompassing residues 1585-1715 (Fig. 10B, upper panel).

To validate these findings and to assess whether the identified domain was also required for binding the other kinases, we determined the impact of deleting residues 1585-1715 from the AKAP-Lbc on its ability to associate with HA-p38α, HA-MKK3, HA-MLTK and endogenous PKNα in co-immunoprecipitation experiments (Fig. 10C-F).

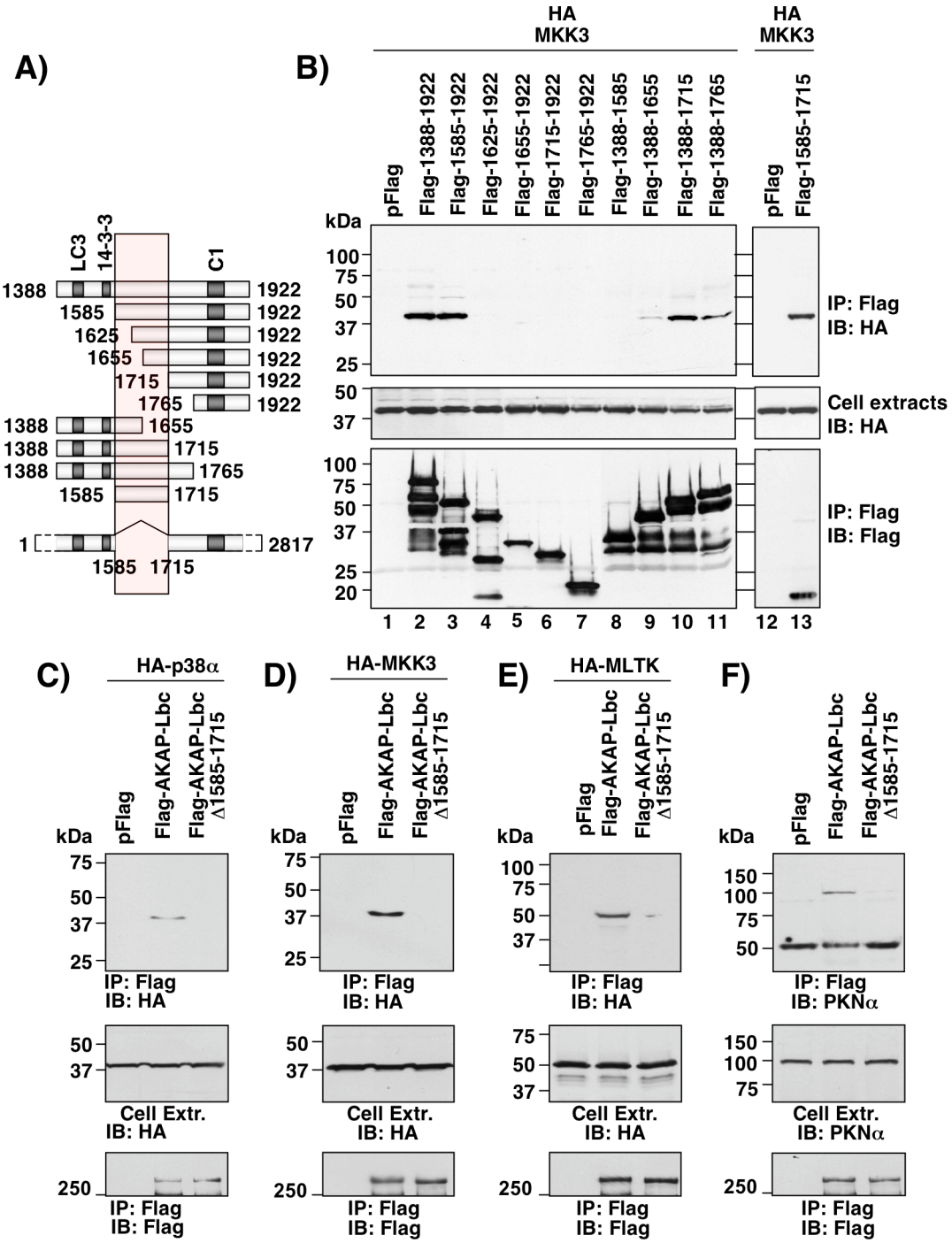


Figure 10: Mapping the kinase interaction sites on AKAP-Lbc. A) Schematic representation of the AKAP-Lbc fragments used for the mapping experiments. The minimal binding site (residues 1585-1715) is boxed. LC3 and 14-3-3 binding sites as well as the C1 region (C1) are shown. **(B)** HEK-293 cells were transfected with HA-tagged MKK3 in combination with either the empty Flag vector or Flag-tagged fragments of AKAP-Lbc indicated in A). Cell lysates were subjected to immunoprecipitation with anti-Flag antibodies. Western blots of the immunoprecipitates and of the cell extracts were revealed using anti-HA polyclonal antibodies to detect the HA-tagged MKK3 (upper and middle panels), or anti-Flag monoclonal antibodies to detect the Flag-tagged AKAP-Lbc fragments (lower panel). **C-F)** Extracts from HEK-293 cells transfected with the plasmids encoding the empty Flag vector, Flag-AKAP-Lbc or the Flag-AKAP-Lbc D1585-1715 mutant in combination with the vectors encoding HA-tagged p38α (C), MKK3 (D) or MLTK (E) or the empty pRK5 vector (F). Western blots of the immunoprecipitates and the cell extracts were revealed using anti-HA polyclonal antibodies to detect HA-tagged p38α, MKK3 and MLTK (C-E, upper and middle panels), anti-PKNα monoclonal antibodies to detect endogenous PKNα (F, upper and middle panels) or anti-Flag monoclonal antibodies to detect Flag-AKAP-Lbc (lower panels).

The deletion reduced significantly the ability of all the tested kinases to co-immunoprecipitate with the flag flag-tagged AKAP-Lbc (Fig. 10C-F, upper panel, lanes 2 and 3). These findings suggest that residues 1585-1715 form a binding site that recruits p38α as well as its upstream activating kinases MKK3, MLTK and PKNα.

4.7 PKNα directly binds AKAP-Lbc, p38α, MKK3 and MLTK

While our current results suggest that AKAP-Lbc interacts with PKNα, MLTK, MKK3 and p38α, they do not indicate how the complex is organized. Based on previous findings showing that PKNα can act both as an upstream activating kinase of MLTK and as a scaffolding protein [215], we hypothesized that it could recruit p38α, MKK3 and MLTK to AKAP-Lbc. Therefore, we determined whether PKNα could directly associate with AKAP-Lbc as well as with the other kinases.

To assess whether the interaction of p38α, MKK3 and MLTK with AKAP-Lbc occurs through a direct interaction or whether it is mediated through PKNα, we monitored the ability of purified GST-fusion proteins of p38α, MKK3, MLTK and of the AKAP-Lbc fragment 1388-1922 to associate with purified His_6-tagged N-terminal or C-terminal fragments of PKNα (His-PKN-1-305 and His-PKN-305-942, respectively), or with the His_6-tagged 1388-1922 fragment of AKAP-Lbc using an in vitro pulldown assay. Interestingly, our results indicate that p38α, MKK3, MLTK as well as AKAP-Lbc can directly bind the C-terminal but not the N-terminal fragment of PKNα (Fig. 11A, upper panel, lanes 3, 5, 7, 9; Fig. 11B, upper panel, lanes 2-6). A weak direct interaction could also be detected between AKAP-Lbc and MKK3 (Fig.11 A, upper panel, lane 6), whereas no binding was observed between AKAP-Lbc and p38α or MLTK (Fig.11 A, upper panel, lane 4 and 8). These results indicate that AKAP-Lbc can directly bind PKNα, which, in turn can recruit p38α, MKK3 and MLTK. The association between AKAP-Lbc and MKK3 could stabilize the formation of the complex.

4.8 PKNα is required for proper assembly of the AKAP-Lbc/p38α signaling complex

To assess whether PKNα could contribute to the assembly of the AKAP-Lbc/p38α complex inside cells, we determined the impact of silencing PKNα expression in HEK-293 cells on the interaction of AKAP-Lbc with p38α and MKK3.

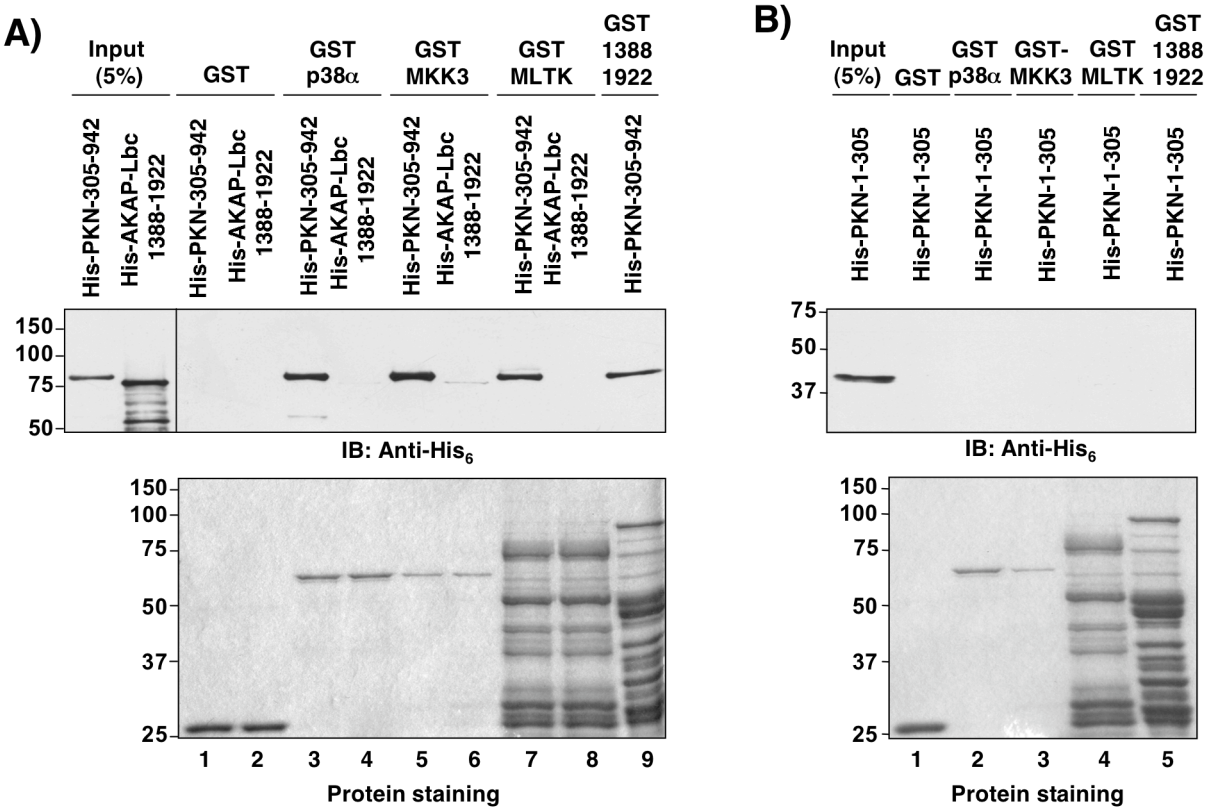


Figure 11. PKNα directly interacts with MLTK, MKK3, p38α and AKAP-Lbc. A) Bacterially purified His_6-tagged fragments (100nM) encompassing residues 305-942 of PKNα and 1388-1922 of AKAP-Lbc were incubated with glutathione-sepharose beads coupled to 2µg of GST alone, or GST-tagged p38α, MKK3, MLTK and AKAP-Lbc 1388-1922. Associated His_6-tagged fragments were detected using anti-His_6 monoclonal antibodies (upper panel). A control protein staining indicating the expression level of the different GST-tagged constructs used in the pulldown assay is shown (lower panel). **B)** Bacterially purified His_6-tagged fragments encompassing residues 1-305 of PKNα (100nM) were incubated with glutathione-sepharose beads coupled to 2µg of GST alone, or GST-tagged p38α, MKK3, MLTK and AKAP-Lbc 1388-1922. Associated His_6-tagged fragments were detected as indicated in A).

PKNα silencing was achieved by infecting cells using lentiviruses encoding shRNAs directed against PKNα. Using this approach, PKNα expression could be inhibited by 80-90% as compared to cells infected with control lentiviruses (Fig. 12B, panel 1).

AKAP-Lbc was immunoprecipitated from infected cells using affinity purified anti-AKAP-Lbc antibodies and the presence of associated p38α and MKK3 revealed by western blot. As shown in Fig. 12A, silencing of PKNα expression significantly reduced the amount of

p38α and MKK3 interacting with endogenous AKAP-Lbc (Fig. 12A, panels 1 and 3, lane 4). This suggests that PKNα favors the association of p38α and MKK3 with AKAP-Lbc.

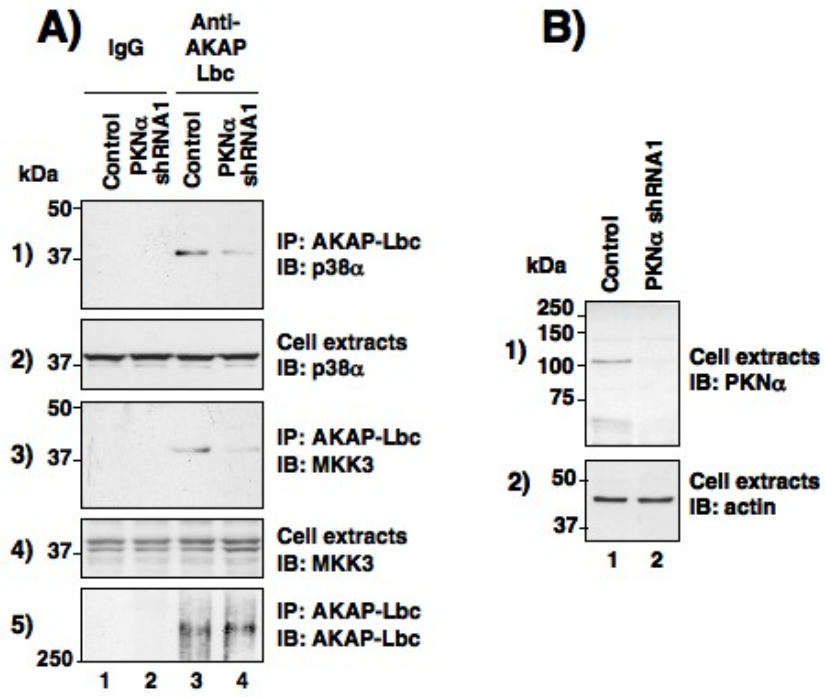


Figure 12 PKN is required for proper assembly of the AKAP-Lbc/p38 signaling complex A) Extracts from HEK-293 cells infected with control lentiviruses (control) or lentiviruses encoding PKNα shRNAs (PKNα shRNA1) were subjected to immunoprecipitation with either non-immune IgGs or affinity purified anti-AKAP-Lbc polyclonal antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using antibodies against p38α (panel 1 and 2), MKK3 (panel 3 and 4), AKAP-Lbc (panel 5). **B)** Western blots of extracts from HEK-293 cells infected with control lentiviruses (control) or lentiviruses encoding PKNα shRNAs (PKNα shRNA1) were revealed using antibodies against the PKNα (panel 6) or actin (panel 7)

4.9 PKNα mediates AKAP-Lbc-induced activation of MLTK

Previous findings have shown that PKNα can directly phosphorylate and activate MLTK [215]. Based on this evidence, we determined whether PKNα was required to transmit activating signals from AKAP-Lbc to MLTK. To address this point, we assessed whether silencing of PKNα using shRNAs targeting two distinct regions of the kinase (PKN shRNA1 and PKN shRNA2) could affect the ability of the S1565A mutant of AKAP-Lbc, which displays constitutively Rho-GEF activity, to activate MLTK.

Infected cells were transfected with the cDNAs encoding Flag-MLTK and the GFP-tagged AKAP-Lbc S1565A mutant. After a 24 h serum starvation, Flag-MLTK was then

immunoprecipitated and its ability to phosphorylate purified GST-MKK3 determined using an *in vitro* kinase assay. Interestingly, silencing of PKNα expression significantly reduced the ability of AKAP-Lbc S1565A to promote MLTK activation (Fig. 13, panel 1, lanes 5 and 6) suggesting that PKNα is required for AKAP-Lbc-mediated MLTK activity.

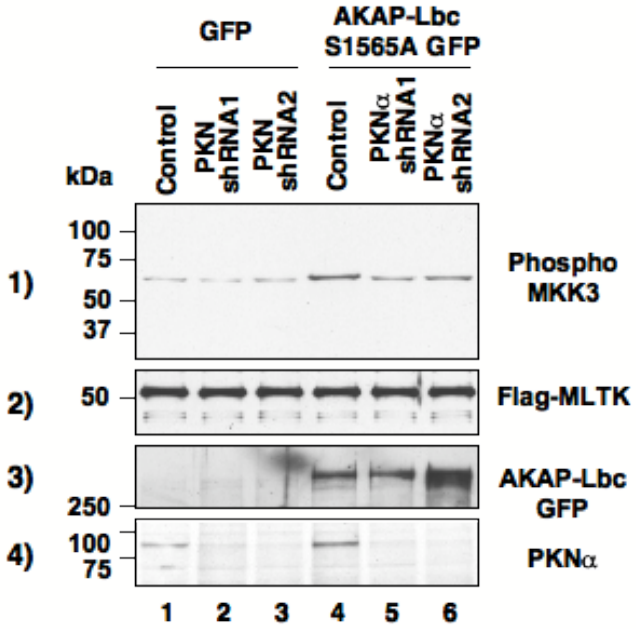


Figure 13 PKNα mediates AKAP-Lbc-induced activation of MLTK HEK-293 cells infected with control lentiviruses or lentiviruses encoding PKNα shRNAs (shRNA1 and shRNA2) were transfected with the plasmids encoding Flag-MLTK in the presence of the vector encoding GFP or the GFP-tagged AKAP-Lbc S1565A mutant. After a 24 h serum starvation, cells were lysed and Flag-MLTK subjected to immunoprecipitation using anti-Flag monoclonal antibodies. Kinase reactions were performed by incubating Flag-MLTK immunoprecipitates with 1 µg of purified GST-MKK3 and in the presence of ATP. Phospho-GST-MKK3 was detected by immunoblot using a rabbit polyclonal antibody recognizing phospho-serine 189 and phospho-threonine 193 of MKK3 (panel 1). The amounts of immunoprecipitated Flag-MLTK as well as the expression of AKAP-Lbc S1565A-GFP and PKNα in cell lysates were assessed using antibodies against the Flag tag (panel 2), GFP (panel 3) and PKNα (panel 4), respectively. Results are representative of three independent experiments.

4.10 Disruption of AKAP-Lbc complex impairs α1-AR-mediated p38α activation

Based on the mapping studies presented above, we investigated the possibility of whether a fragment of AKAP-Lbc encompassing residues 1585-1715 could be used as a competitor to disrupt the endogenous complexes formed by AKAP-Lbc and the various kinases. Such a competitor fragment could represent a valuable tool to study the role of the AKAP-Lbc signaling complex in the activation of p38α inside cells.

A GFP fusion of the competitor fragment was expressed in HEK-293 cells and its ability to inhibit the binding of HA-MKK3, HA-MLTK as well as PKNα to the Flag-tagged fragment of AKAP-Lbc encompassing residues 1388-1922 was assessed by co-immunoprecipitation (Fig. 14). Interestingly, overexpression of the fragment reduced the interaction between AKAP-Lbc and the various kinases by more than 80% suggesting that it can act as an efficient competitive inhibitor (Fig. 14A-C, upper panel, lane 3).

Based on these results we determined the impact of overexpressing the GFP-tagged competitor fragment on the ability of α1b-adrenergic receptors to induce p38α activation in HEK-293 cells.

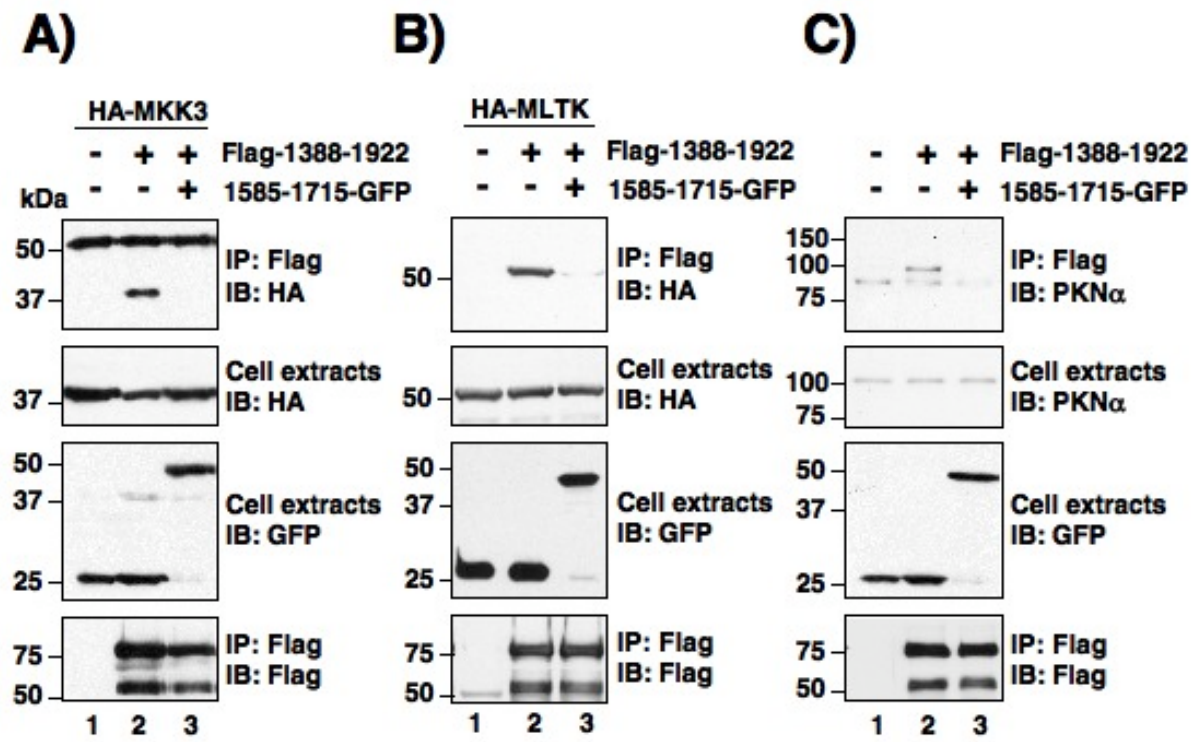


Figure 14: The fragment of AKAP-Lbc encompassing residues 1585-1715 acts as a competitive inhibitor of the association of MKK3, MLTK and PKNα with AKAP-Lbc. A-C) Extracts from HEK-293 cells transfected with the plasmids encoding HA-MKK3 (A), HA-MLTK (B) or the pRK5 vector (C) together with the empty Flag vector and the pEGFP plasmid (lane 1), the vector encoding the Flag-tagged AKAP-Lbc fragment encompassing residues 1388-1922 and the pEGFP plasmid (lane 2) or a combination of the vectors encoding the Flag-tagged 1388-1922 fragment and the GFP-tagged fragment of AKAP-Lbc encompassing residues 1585-1715 (lane 3), were subjected to immunoprecipitation with anti-Flag antibodies. Western blots of the immunoprecipitates and cell extracts were revealed using anti-HA to detect HA-MKK3 (A, upper panel) and HA-MLTK (B, upper panel), anti-PKNα antibodies to detect PKNα (C, upper panel), anti-GFP antibodies to detect the GFP-tagged 1585-1715 fragment (middle panels), or anti-Flag antibodies to detect the Flag-tagged 1388-1922 fragments (lower panels).

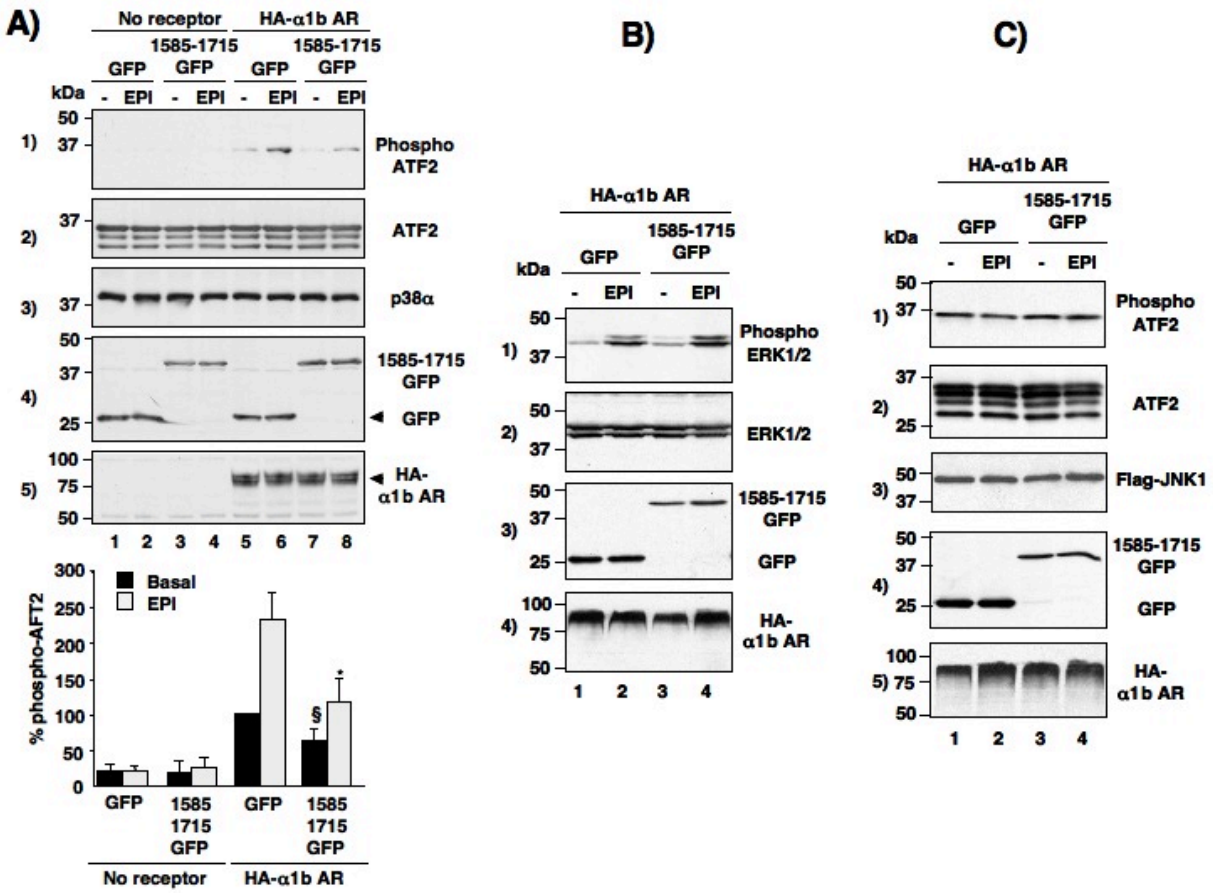


Figure 15. Disruption of the AKAP-Lbc complex inhibits α1b-AR-induced p38α activation. A) HEK-293 cells were transfected with the vectors encoding GFP or the GFP-tagged AKAP-Lbc fragment 1585-1715 in the absence or presence of the plasmid encoding the HA-tagged α1-AR. After a 24 h serum starvation, cells were incubated for 15 min with or without 10^{-4} M epinephrine (EPI), lysed and subjected to immunoprecipitation using anti-p38α monoclonal antibodies. Kinase reactions were performed by incubating p38α immunoprecipitates with 1 µg of purified GST-ATF2 and in the presence of ATP. Detection of phospho-ATF2 (panel 1), ATF2 (panel 2), p38α (panel 3) and HA-tagged α1-AR (panel 5) were performed as indicated in Fig. 1A. Expression of GFP as well as the GFP-tagged AKAP-Lbc fragment 1585-1715 (panel 4) was detected using polyclonal anti-GFP antibodies. **B)** HEK-293 cells were transfected with the vectors encoding GFP or the GFP-tagged AKAP-Lbc 1585-1715 in the presence of the plasmid encoding HA-tagged α1b-AR. After a 24 h serum starvation, cells were incubated for 15 min with or without 10^{-4} M epinephrine (EPI) and lysed. Phosphorylation of ERK1/2 was assessed by western blot using polyclonal antibodies recognizing phospho-ERK1/2 (panel 1). The amounts of ERK1/2, GFP, GFP-tagged AKAP-Lbc 1585-1715 and HA-α1b-ARs in the cell lysates were assessed using polyclonal antibodies against ERK1/2 (panel 2), GFP (panel 3), the HA epitope (panel 4), respectively. Results are representative of 3 different experiments. **C)** HEK-293 cells were transfected with the vectors encoding GFP or the GFP-tagged AKAP-Lbc fragment 1585-1715 and in the presence of the plasmids encoding HA-tagged α1b-AR and Flag-JNK1. After a 24 h serum starvation, cells were incubated for 15 min with or without 10^{-4} M epinephrine (EPI), lysed and subjected to immunoprecipitation using monoclonal anti-Flag antibodies. The kinase activity of immunoprecipitated JNK1 was assessed in vitro by monitoring its ability to induce the phosphorylation of ATF2. The amounts of phospho-ATF2, ATF2, Flag JNK1, GFP-tagged AKAP-Lbc 1585-1715, and HA-tagged α1b-AR were assessed using antibodies against phospho ATF (panel 1), ATF2 (panel 2), the Flag epitope (panel 3), GFP (panel 4), and the HA-tag (panel 5), respectively.
Quantitative analysis of phosphorylated ATF2 was obtained by densitometry. The amount of phospho-ATF2 was normalized to the total amount of ATF2. Results are expressed as mean ± S.E. of 3 different experiments.). § $p < 0.05$ as compared with phospho-ATF2 levels measured in untreated cells expressing HA-α1b-ARs and GFP. * $p < 0.05$ as compared with phospho-ATF2 levels measured in epinephrine-treated cells expressing HA-α1b-ARs and GFP.

Interestingly, expression of the competitor fragment reduced by 62% and 58% the ability of α1b-ARs to promote p38α activation under basal conditions and in response to epinephrine stimulation, respectively (Fig. 15A, panel 1, lanes 7 and 8;), without affecting receptor-induced phosphorylation of ERK1/2 and JNK1 (Fig. 15B-C, panel 1, lanes 3 and 4). These findings suggest that the integrity of the complex formed by AKAP-Lbc and the various kinases is required for the activation of p38α induced by α1b-ARs.

4.11 Binding of 14-3-3 to AKAP-Lbc inhibits the recruitment of PKNα and reduces p38α activation

Our laboratory previously demonstrated that recruitment of the regulatory protein 14-3-3 to a motif located at position 1565 within the N-terminal regulatory region of AKAP-Lbc strongly inhibits the Rho-GEF activity of the anchoring protein [195]. Our current results indicate that this site is located in close proximity of the binding domain for the p38α activation complex. This raises the possibility that 14-3-3 recruitment could also interfere with the interaction of AKAP-Lbc with the p38α signaling complex and therefore inhibit AKAP-Lbc-mediated p38 activation.

To address this question, we initially determined whether the overexpression of 14-3-3β in HEK-293 cells could affect the ability of AKAP-Lbc to associate with PKNα. HEK-293 cells were transfected with Flag AKAP-Lbc together with increasing amounts of GFP-tagged 14-3-3β. After immunoprecipitating the anchoring protein using anti-Flag antibodies, anti-PKNα antibodies were used to immunoblot the immunoprecipitated samples. Western blots revealed that overexpression of increasing amount of 14-3-3β progressively reduced the ability of PKNα to co-immunoprecipitate with AKAP-Lbc (Fig. 16A, panel 1, lanes 2-4;). In line with these results, we could show that the S1565A mutant of AKAP-Lbc, which is unable to bind 14-3-3, display a 2 fold higher ability to associate with endogenous PKNα when compared to wild type AKAP-Lbc (Fig. 16B, panel 1, lanes 2 and 3). This indicates that recruitment of 14-3-3 inhibits PKNα binding to AKAP-Lbc.

To determine whether this reduction of PKNα binding induced by 14-3-3 could affect the ability of AKAP-Lbc to induce p38α activation, we compared the p38 activating potential of the wild type and 14-3-3 binding deficient forms of AKAP-Lbc. We could show that deletion of the 14-3-3 binding site increases by 2.7 folds the activation of p38α induced by the anchoring protein (Fig. 16C, upper panel, lanes 2 and 3). Collectively, these results

suggest that 14-3-3 exerts an inhibitory effect on the ability of AKAP-Lbc to recruit and activate the p38α signaling complex.

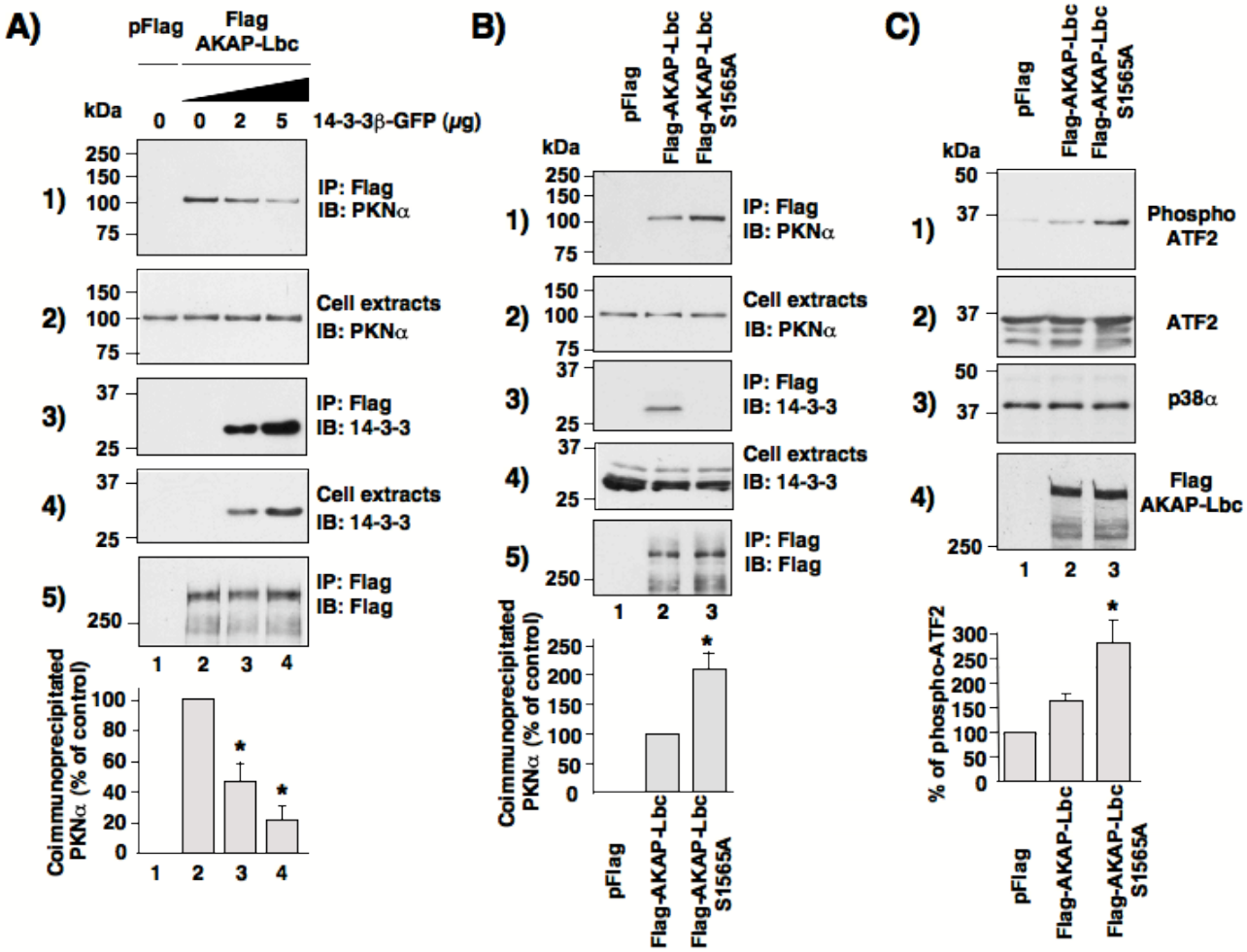


Figure 16) 14-3-3 inhibits the interaction between AKAP-Lbc and PKNα. A) HEK-293 cells were transfected with the empty pFlag vector or the vector encoding Flag-AKAP-Lbc in combination with increasing amounts (indicated above each lane) of the plasmid encoding 14-3-3β -GFP. Cell extracts were subjected to immunoprecipitation with anti-Flag antibodies. Western blots of the immunoprecipitates and of the cell extracts were revealed using anti-PKNα polyclonal antibodies (panels 1 and 2), anti-14-3-3β polyclonal antibodies (panels 3 and 4) or anti-Flag monoclonal antibodies to detect the Flag-AKAP-Lbc (panel 5). Densitometry of the bands corresponding to PKNα coimmunoprecipitated with AKAP-Lbc. The amount of PKNα in the immunoprecipitates was normalized to the PKNα content of the cell extracts. Results are expressed as mean± S.E. of three independent experiments. * $p < 0.05$ as compared with the levels of co-immunoprecipitated PKNα measured in cells expressing only Flag-AKAP-Lbc. **B)** Extracts from HEK-293 cells transfected with the plasmids encoding the empty Flag vector, Flag-AKAP-Lbc or the Flag-AKAP-Lbc S1565A. Cell extracts were subjected to immunoprecipitation with anti-Flag antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed as indicated in A). Densitometry of the bands corresponding to PKNα coimmunoprecipitated with AKAP-Lbc was performed as indicated in B). Results are expressed as mean± S.E. of three independent experiments. * $p < 0.05$ as compared with the levels of co-immunoprecipitated PKNα measured in cells expressing Flag-AKAP-Lbc. **C)** Extracts from HEK-293 cells transfected with the vectors encoding Flag AKAP-Lbc-GFP or Flag-AKAP-Lbc S1565. After a 24 h serum starvation, cells were lysed and subjected to immunoprecipitation using monoclonal anti-p38α antibodies. Kinase reactions and detection of phospho-ATF2 (panel 1), ATF2 (panel 2) and p38α (panel 3) were performed as indicated in Fig. 1A. Expression of the AKAP-Lbc constructs was detected using anti-Flag monoclonal antibodies (panel 4). Quantitative analysis of phospho-ATF2 was obtained as indicated in Fig. 1B. Results are expressed as mean ± S.E. of three different experiments. * $p < 0.05$ as compared with phospho-ATF2 levels measured in cells expressing Flag-AKAP-Lbc.

V DISCUSSION

Here, we report that AKAP-Lbc organizes a molecular signaling complex that is required to mediate the p38 MAP kinase activation downstream of α1b-adrenergic receptors.

MAP kinase pathways are crucial mediators of several pathophysiological responses induced by α1-adrenergic receptors [23, 211-213]. In particular p38 kinases were originally described to mediate cellular responses to various types of stresses [208, 214]. During the last years, however, it has become increasingly clear that members of this kinase family can participate in signaling pathways activated by a variety of other membrane receptors, including cytokine and G protein coupled receptors, to promote cellular functions such as proliferation, growth, inflammation, and contraction [208, 214]. Interestingly, it was shown that activation of p38α by α1-ARs could regulate arterial smooth muscle cell contractility [215] and promote cardiomyocyte sarcomere remodeling during cardiac hypertrophy [212].

It is now clear, by different lines of evidence collected over the last years that these signaling cascades are organized in transduction modules [216], but it is currently unknown how such signaling complexes are assembled and get activated in response to α1-adrenergic receptor stimulation to generate specific cellular responses.

In this study we have demonstrated that the RhoA-selective exchange factor, AKAP-Lbc, recruits a signaling module containing the RhoA effector PKNα and the kinases MLTK, MKK3 and p38, and it transduces activating signals from α1ARs down to p38 (Fig. 17)

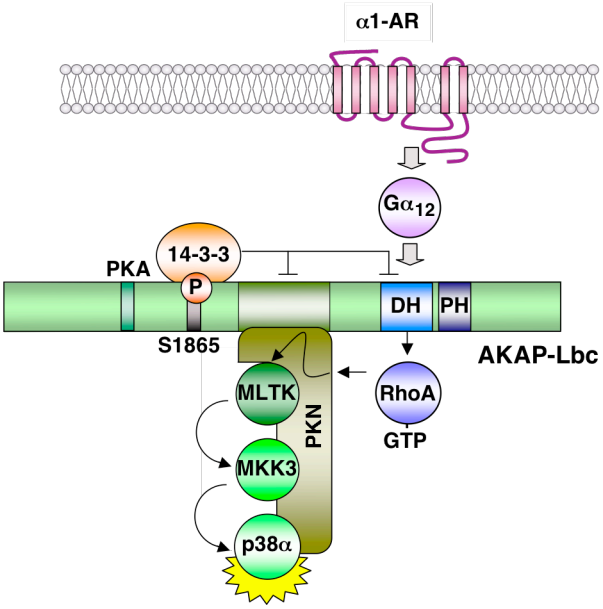


Figure 17) Model for the AKAP-Lbc-p38α activation complex. AKAP-Lbc assembles a signaling complex which includes the scaffolding protein PKNα as well as MLTK, MKK3 and p38α. The AKAP-Lbc signaling complex is activated in response to α1-AR stimulation through a Gα12 mediated signaling pathway. Activated AKAP-Lbc promotes the formation of RhoA-GTP, which, in turn, induces the activation of a signaling cascade that includes PKNα, MLTK, MKK3 and p38α. The recruitment of 14-3-3, inhibits AKAP-Lbc Rho-GEF activity, impairs the interaction between PKNα and AKAP-Lbc, and reduces p38 activation.

In this context, it is well established that GTPases of the Rho family, including RhoA, Rac1, and Cdc42, are key mediators of p38 activation induced by membrane receptors [209, 212, 217, 218]. However, how activation of Rho GTPases by upstream stimuli is translated into the activation of a specific p38 pathway is not clear.

Similar to our findings, two previous studies have characterized that the GTPase Rac-1 and its upstream activator Tiam can recruit signaling complexes containing p38 and its upstream activating kinases. In the first study, Rac-1 was shown to directly bind to Osm, a scaffold protein that recruits MEKK3 and MKK3 [219]. This complex has been implicated in hyperosmotic shock-induced p38 activation. In a second study, the Rac-1 activator Tiam was shown to recruit a p38 signaling complex formed by the scaffold protein JIP2 and the kinases MLK3 and MKK3 [220]. This study, however, did not determine which extracellular stimuli activate this signaling module or whether Tiam and JIP2 can form a complex at the endogenous level.

More recently, elegant studies showed that the pro-myogenic cell surface protein Cdo can recruit two proteins named Bnip-2 and JLP, which act as scaffolds for the Cdc42 and p38, respectively [221, 222]. The assembly of this signaling complex promotes Cdc42–dependent p38 activation.

In our project AKAP-Lbc can mediate α1-AR-induced p38 activation through the assembly of a signaling module composed of PKNα, MLTK, MKK3 and p38α. Interestingly our results indicate that deletion of the PKNα binding site (residues 1585-1715) from AKAP-Lbc abolishes the ability of the anchoring protein to recruit the p38α and its upstream kinases MKK3 and MLTK. These results suggest that PKN in our model is not only an effector of RhoA but it acts as a scaffold protein that organizes a p38 signaling cascade to facilitates RhoA-dependent p38 activation.

In line with our observations, the potential scaffold role of PKN was already described ten years ago by an interesting paper, which has demonstrated that PKN could promote the activation of p38γ though the specific recruitment of MLTK and MKK6. These results raise the hypothesis that PKN, depending on the cell types, can recruit different combinations of signaling enzymes to modulate the activation of different p38 isoforms. In our project the co-immunoprecipitation experiments failed to detect an interaction between p38γ and AKAP-Lbc (results not shown), suggesting that AKAP-Lbc might selectively stabilize the interaction between PKNα and p38α.

Currently, three other scaffold proteins including JIP2, JIP4 and Osm have been shown to promote p38 activation [216, 219, 223]. Interestingly, recent studies identified an interaction between recombinant JIP4 and a splice variant of AKAP-Lbc, called Brx, that contains only the last 1429 residues the anchoring protein [224]. However, while these studies mapped the interaction determinants for JIP4 to the last 400 amino acids of Brx, they did not determine whether the two proteins are able to interact at the endogenous level. In our control experiments, we could not detect the expression of JIP4 in HEK-293 cell lysates (results not shown), suggesting that JIP4 is unlikely to be involved in the recruitment of p38α and its upstream kinases to AKAP-Lbc in this cell line.

Altogether these results provide new mechanistic insights on how specific signals can be driven from membrane receptors to p38α.

This conclusion has been supported by the fact that AKAP-Lbc specifically mediates α1-AR-induced p38α activation without affecting activation of ERK1/2 and JNK. This was demonstrated by the co-immuoprecipitation experiments that did not show any interaction between ERK1/2 and JNK with AKAP-Lbc.

In addition, our results suggest the presence of other signaling complexes that activate ERK1/2 and JNK in response to α1-AR-induced. In this context, a previous study has shown that the Rho-GEF p115 can recruit a scaffolding protein named CNK1, which binds the kinases MLK2 and MKK7, to coordinate the activation of JNK1 [225]. However, further investigations will be required to determine whether p115 or a different signaling complex is involved in the organization of the JNK pathway downstream of α1b-ARs.

By mapping the interaction between AKAP-Lbc and PKN we could see that the PKN binding site (1585-1715) is located in proximity of the 14-3-3 binding site (serine 1565). The members of the 14-3-3 family are well characterized to negatively regulate a variety of transduction pathways including the p38 activation pathway [226, 227]. Interestingly, previous observations from our lab have demonstrated that 14-3-3 impairs the Rho-GEF activity of AKAP-Lbc. In this context, our current findings suggest that 14-3-3 can inhibit AKAP-Lbc-mediated p38α activation in two ways: by inhibiting the Rho-GEF activity of ALKAP-Lbc [42] and by inducing the dissociation of PKNα-MLTK-MKK3-p38α complex from AKAP-Lbc (Fig. 8).

The second mechanism inhibiting p38 activation could be explained by the fact that 14-3-3 recruitment might block the interaction between AKAP-Lbc and PKNα by directly masking the PKNα interaction site on the anchoring protein. This is reminiscent of the mechanism

through which 14-3-3 has been shown to inhibit the interaction between the pro-apoptotic protein Bad and pro-survival Bcl-2 family members [228].

In conclusion, the implications of our findings are twofold. Firstly, they identify key molecular mechanisms controlling signaling specificity downstream of α1b-ARs. By assembling a macromolecular signaling complex containing RhoA, PKNα, MLTK, MKK3 and p38α, AKAP-Lbc controls the specific transduction of signals from α1b-ARs to p38α. Secondly, they provide a novel hypothesis explaining the inhibitory action of 14-3-3 on the p38 pathway, suggesting that AKAP-Lbc might represent a molecular platform integrating 14-3-3 and p38 signaling.

VI. RESULTS II: Characterization of novel hypertrophic pathway activated by the AKAP-Lbc signaling complex in cardiomyocytes

5.1 AKAP-Lbc assembles a p38α activation module in cardiomyocytes

Our previous results have shown that AKAP-Lbc forms a stable complex with p38α and its upstream kinases MKK3, MLTK and PKNα in HEK 293 cells. Moreover our results indicate that the formation of this complex is required for the ability of AKAP-Lbc to activate p38α.

To determine whether endogenous AKAP-Lbc can form a stable complex with these different kinases in cardiomyocytes, we immunoprecipitated AKAP-Lbc from RNVMs, using an affinity purified anti-AKAP-Lbc specific antibody, and assessed the presence of associated kinases by immunoblot using specific antibodies. Our results indicate that p38α, MKK3, MLTK and PKNα endogenously expressed in cardiomyocytes could specifically co-immuno-precipitate with AKAP-Lbc (Fig. 1).

Interestingly, only the 39 kDa form of MKK3 (Isoform B [216]) (Fig. 1A, panel 2 lanes 1-3) and the 75 kDa of MLTK (MLTK beta [217]) (Fig. 1B panel 1, lanes 1-3) could form a complex with the anchoring protein. On the other hand, no interaction could be detected between the endogenous AKAP-Lbc with MLK3 (Fig. 1B panel 2, lanes 1-3).

Altogether, these results suggest that AKAP-Lbc specifically interact with p38α and its upstream kinases MKK3β, MLTKβ and PKNα to form a stable complex in cardiomyocytes.

5.2 AKAP-Lbc is involved in α1-AR-mediated p38 activation in cardiomyocytes

Our previous experiments performed in HEK-293 cells have demonstrated that AKAP-Lbc could activate the kinase p38 in response to α1-ARs stimulation.

To provide a formal proof that AKAP-Lbc mediates the activation of p38α in cardiomyocytes, we silenced AKAP-Lbc expression in RNVMs using lentiviral vectors

encoding AKAP-Lbc specific shRNAs and determined whether this could affect the phosphorylation of p38α induced by the α1-ARs.

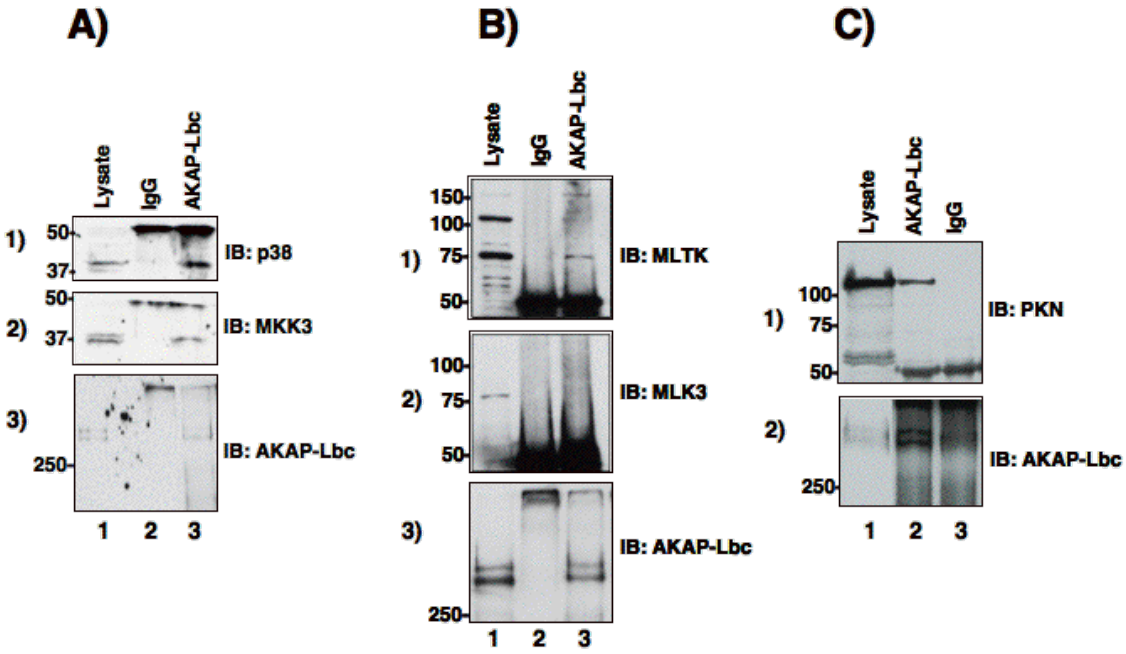


Figure 1. AKAP-Lbc interacts with endogenous p38α, MKK3β, MLTKβ and PKNα. NRVMs cell extracts were subjected to immunoprecipitation with either non-immune IgGs or affinity purified anti-AKAP-Lbc polyclonal antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using anti-p38α (A, upper panel), anti-MKK3 (A, middle panel), anti-MLTK (B, upper panel), anti-MLK3 (B, middle panel) anti-PKNα (C, upper panel) or affinity purified anti-AKAP-Lbc polyclonal antibodies (lower panels). Results are representative of at least three independent experiments.

Indeed, we could show that AKAP-Lbc silencing reduced the ability of α1-ARs to induce the phosphorylation of p38 (Fig.2, panel 1, lane 4), while in control experiments, infection of RNVMs with lentiviral vectors encoding mutated shRNAs did not affect p38α phosphorylation (Fig2, panel 1 lanes 2 and 6).

These results indicate that, in cardiomyocytes, AKAP-Lbc transduces signals involved in the activation of the p38 in response to the stimulation of α1-ARs.

5.3 The AKAP-Lbc/p38 complex is involved in α1-AR-mediated GATA4 activation in cardiomyocytes

As reported in the introduction, p38 has a relevant role in activating transcription factors that control the transcription of genes involved in cardiac hypertrophy such as GATA-4 and MEF2C though direct phosphorylation of specific serine residues located within their transactivation domains (Ser 105 of GATA-4, Ser 59 of MEF-2C). In addition, an important study has demonstrated that RhoA potentiates the transcriptional activity of GATA-4 via a

p38-dependent pathway that phosphorylates GATA-4 activation domains and GATA binding sites [141].

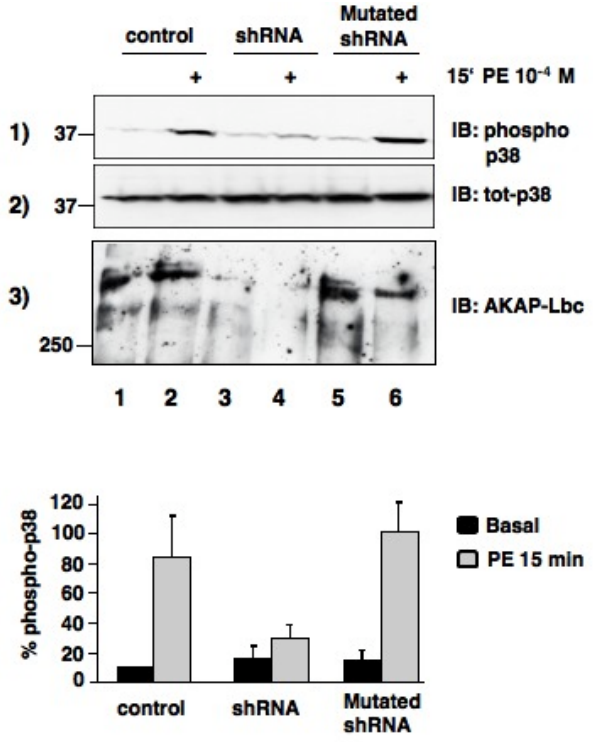


Figure 2 Silencing of AKAP-Lbc expression inhibits α1-AR-mediated phosphorylation of p38: RNVMs were infected with lentiviruses encoding GFP (control), GFP-wild-type and GFP-mutated AKAP-Lbc shRNAs at a moi of 50. Four days after infection, cardiomyocytes serum starved, were incubated for 15 min with or without (Ctrl) 10^{-4} M phenylephrine (PE). Proteins in the cell lysates were separated by SDS-PAGE and the phosphorylated form of p38, was detected using phospho specific antibody of p38, (upper panel). The amounts of total p38, in the cell lysates were assessed using specific antibodies against p38, (middle panel). The amounts of total AKAP-Lbc in the cell lysates was assessed using polyclonal antibodies against the AKAP-Lbc (lower panel). Quantitative analysis of phosphorylated p38 was obtained by densitometry. The amount of phospho-p38 was normalized to the total amount of p38α. Results are expressed as mean ± S.E. of 6 different experiments.

In order to determine whether AKAP-Lbc silencing could inhibit the activity of GATA4 in response to the stimulation of α1-ARs, RNVMs have been infected with lentiviral expression vectors encoding shRNAs directed against AKAP-Lbc and subsequently transfected with the plasmid encoding the firefly luciferase under the control of GATA4 responsive elements. The activity of GATA4 was measured by dual luciferase assay.

Interestingly, GATA4 transcriptional activity induced by the stimulation of α1-ARs was inhibited by 65% following AKAP-Lbc silencing, as compared to the control cells (Fig. 3B).

Using the same assay we could show that p38 inhibition using the specific p38 inhibitor SB203580 could completely block α1-AR-induced GATA4 activation.

These results suggest that AKAP-Lbc/p38α complex can activate the transcription factor GATA4 in response to the stimulation of α1-ARs in NRVMs.

Notably it will important to determine whether the AKAP-Lbc/p38 complex contributes to the activation of GATA4 through the phosphorylation of serine 105 in response to α1-AR stimulation.

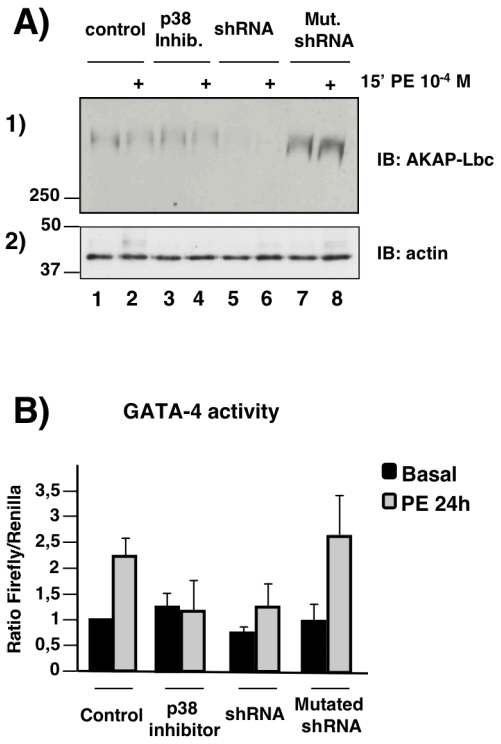


Figure 3 Silencing of AKAP-Lbc expression inhibits α1-AR-mediated activation of GATA4: A) RNVMs were infected with lentiviruses encoding GFP (control), GFP-wild-type and GFP-mutated AKAP-Lbc shRNAs at a moi of 50. Four days after infection, cardiomyocytes serum starved, were incubated for 15 min with or without (Ctrl) 10^{-4} M phenylephrine (PE). Proteins in the cell lysates were separated by SDS-PAGE and the amount of total AKAP-Lbc in the cell lysates was assessed using polyclonal antibodies against the AKAP-Lbc (upper panel). **B)** RNVMs were infected with lentiviruses encoding GFP (control), GFP-wild-type and GFP-mutated AKAP-Lbc shRNAs at a moi of 50. Twenty-four hours after infection cells were co-transfected with GATA4-Luciferase (Firefly) reporter gene plasmid and pcmv-Renilla-Luciferase. Forty-eight hours after transfection cells were incubated for 24 hours with or without (Ctrl) 10^{-4} M phenylephrine (PE). Firefly activity was measured and normalized to Renilla activity. Luciferase activity measured in cells not stimulated was given an arbitrary value of 1.

5.4 AKAP-Lbc signaling complex controls the expression of different hypertrophic genes that are under the control of GATA4

It is well described that GATA4 regulates the transcription of some hypertrophic genes such as the Atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), α-skeletal actin and α cardiac actin. To determine whether the AKAP-Lbc/p38α complex can control the expression of these genes, we have determined the impact of silencing AKAP-Lbc expression in RNVMs using lentiviral expression vectors encoding specific shRNAs on the expression of ANF, β-MHC, α-skeletal actin and α-cardiac actin induced by the stimulation of α1-ARs. To address this issue the expression of these genes has been measured by real time PCR.

Interestingly, our results show that AKAP-Lbc silencing reduces by 50% the expression of ANF and by 35% the expression of β-MHC and α-skeletal actin in response to α1-AR stimulation compare as to the controls while the expression of α cardiac actin was not influenced (Fig. 4).

These results suggest that the AKAP-Lbc signalling complex controls the expression of different hypertrophic genes that are under the control of GATA4.

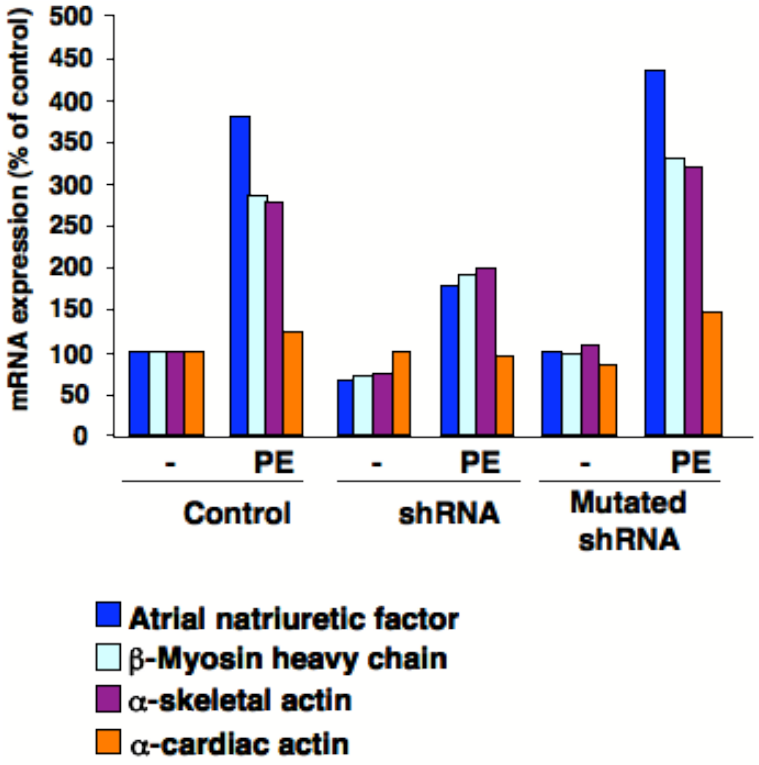


Figure 4 Silencing of AKAP-Lbc expression inhibits α1-AR-mediated induction of hypertrophic genes transcribed by GATA4: RNVMs were infected with lentiviruses encoding GFP (control), GFP-wild-type and GFP-mutated AKAP-Lbc shRNAs at a moi of 50. Seventy-two hours after infection, cells were incubated for 24 h in the absence or presence of 10^{-4} M PE. Real-time PCR analysis of atrial natriuretic factor (ANF) skeletal α-actin (Ska), β Myosin heavy chain and α-cardiac actin mRNA expression was performed on total RNA samples extracted from rat ventricular cardiomyocytes.

5.5 The AKAP-Lbc/p38 complex is involved in α1-AR-mediated Hsp27 activation in cardiomyocytes

It is well established that the MAPK p38 cascade leads to the activation of the kinase MK2, which directly phosphorylates the heat shock protein 27 (Hsp27). Interestingly phosphorylated Hsp27 interacts with actin and intermediate filaments and it protects them from fragmentation. In cardiomyocytes, Hsp27 is phosphorylated in response to the activation of the p38 pathway induced by several stresses. Specifically, phospho-Hsp27 has been shown to bind and stabilize both actin cytoskeleton and sarcomeres.

Knowing the role of cytoskeleton remodelling and sarcomere reorganisation during cardiomyocyte hypertrophy, it is of special interest to characterize the role AKAP-Lbc/p38 complex in the activation of Hsp27 in NRVMs.

To address this issue we assessed whether silencing AKAP-Lbc expression using lentiviral–delivered shRNAs, could significantly impair the phosphorylation of Hsp27 in response to the stimulation of α1ARs, as assessed using Hsp27 phospho-specific antibodies.

Interestingly, our results indicate that inhibition of AKAP-Lbc expression can reduce by 65% the phosphorylation of Hsp27 in response to α1-ARs as compared to the controls (Fig. 5, panel 1, lane 6). In addition, in the same experiments we have shown that p38 inhibition using specific p38 inhibitor SB203580, can completely block the α1-ARs-induced Hsp27 phosphorylation.

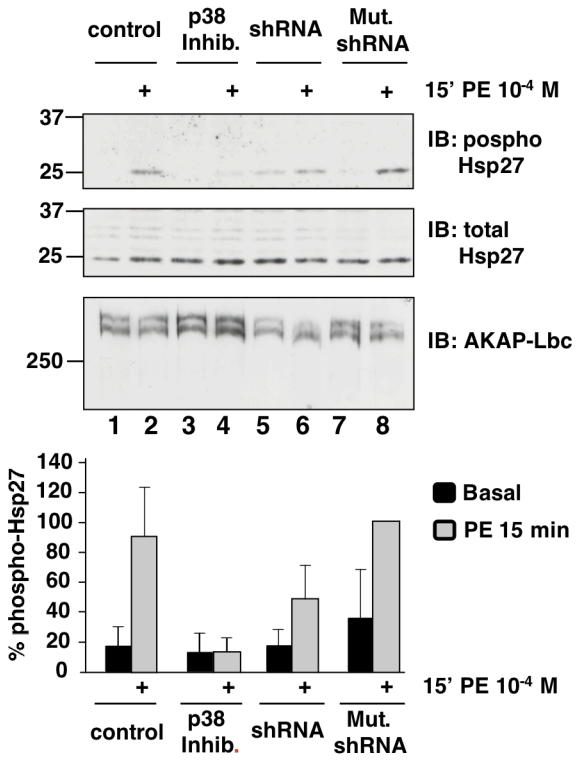


Figure 5 Silencing of AKAP-Lbc expression inhibits α1-AR-mediated phosphorylation of Hsp27: RNVMs were infected with lentiviruses encoding GFP (control), GFP-wild-type and GFP-mutated AKAP-Lbc shRNAs at a moi of 50. Four days after infection, cardiomyocytes serum starved, were incubated for 2h with or without (Ctrl) 10^{-5} M SB203580. Two hours later cardiomyocytes serum starved were incubated with or without (Ctrl) 10^{-4} M phenylephrine (PE). Proteins in the cell lysates were separated by SDS-PAGE and the phosphorylated form of Hsp27, was detected using phospho specific antibody of Hsp27, (upper panel). The amounts of total Hsp27, in the cell lysates were assessed using specific antibodies against Hsp27, (middle panel). The amounts of total AKAP-Lbc in the cell lysates was assessed using polyclonal antibodies against the AKAP-Lbc (lower panel). Quantitative analysis of phosphorylated Hsp27 was obtained by densitometry. The amount of phospho-Hsp27 was normalized to the total amount of Hsp27. Results are expressed as mean ± S.E. of 6 different experiments.

This suggests that AKAP-Lbc/p38 complex promotes the activation of the Hsp27 in response to α1-ARs stimulation, which may in turn stabilize cardiomyocyte cytoskeleton in response to hypertrophic stimuli.

5.6 Hsp27 relocalizes to the cytoskeleton and to the sarcomere in response to α1-ARs stimulation in RNVMs

To futher evaluate the potential role of Hsp27 in α1-Ars-induced cardiomyocyte remodelling, we have determined whether stimulation of RNVMs with PE could promote re-localization of Hsp27 from the cytosol to the cytoskeleton or to sacomeric components, as assessed by immunohystochemistry.

Our results indicate that following 1 hour PE treatment, Hsp27 relocalizes to peripheral actin-positive filaments structures (Fig.6A-B).

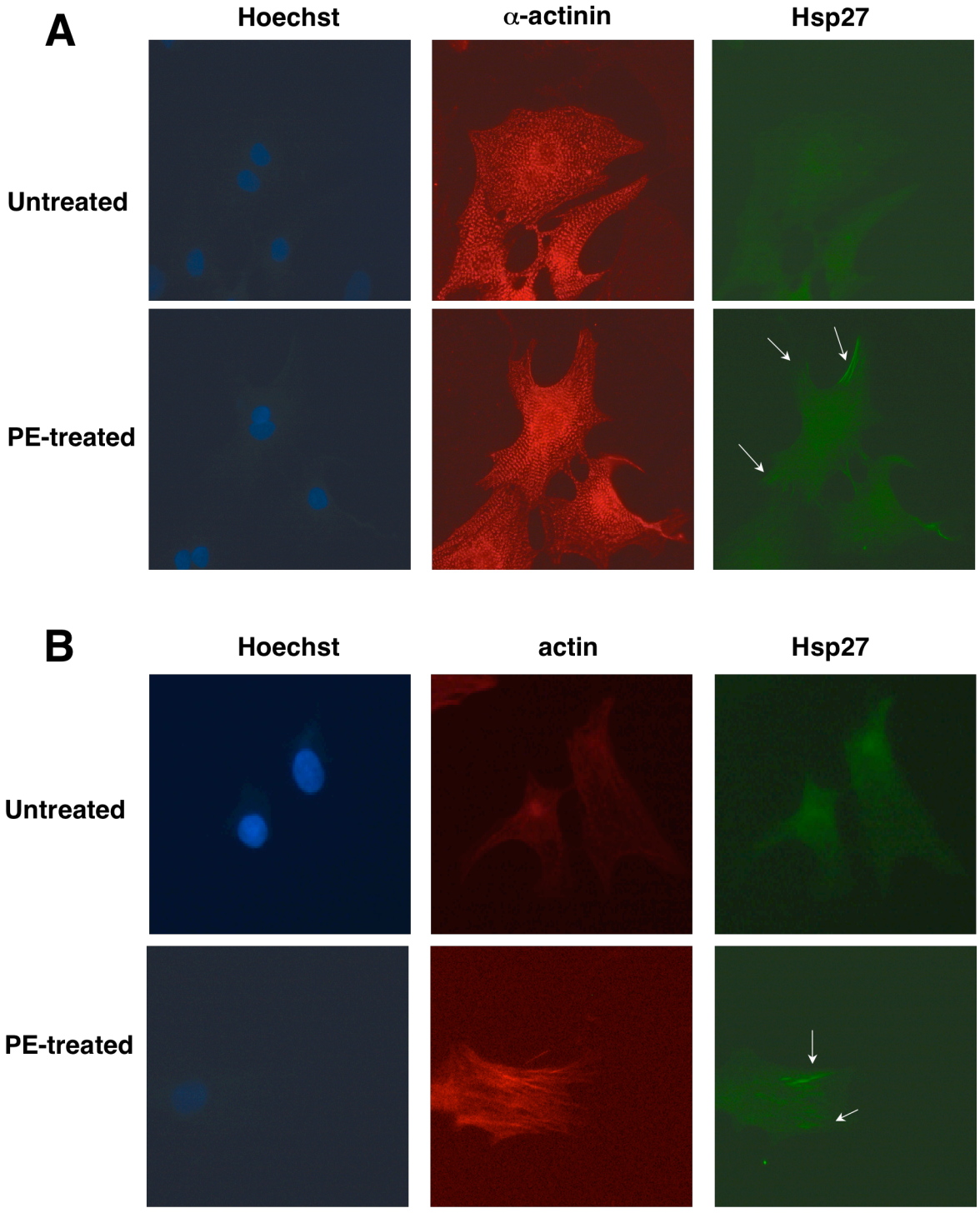


Figure 6 Hsp27 relocalize to the cytoskeleton after stimulation of the α1-AR in RNVMs A) RNVMs were serum starved for 24h. After the starvation RNVMs were incubated for 1 h in the absence or presence of PE. Cells were then fixed, permeabilized, and incubated with anti-Hsp27 polyclonal antibodies as well as FITC conjugated anti-rabbit secondary antibodies and with with anti-α-actinin (red) monoclonal antibodies as well as rhodamine-conjugated anti-mouse secondary antibodies.
B) RNVMs were serum starved for 24h. After the starvation RNVMs were incubated for 1 h in the absence or presence of PE. Cells were then fixed, permeabilized, and incubated with anti-Hsp27 polyclonal antibodies as well as FITC conjugated anti-rabbit secondary antibodies and with rhodamine- conjugated phalloidine to detect the expression of the actin.

Moreover, in some cardiomyocytes PE stimulation induced Hsp27 to re-localize in the sarcomeric proteins.

These results raise the hypothesis that Hsp27 might participate in the reorganization and/or stabilization of sarcomeres and cytoskeleton, following hypertrophic signals. Future experiments using Hsp27 specific shRNA will address this question.

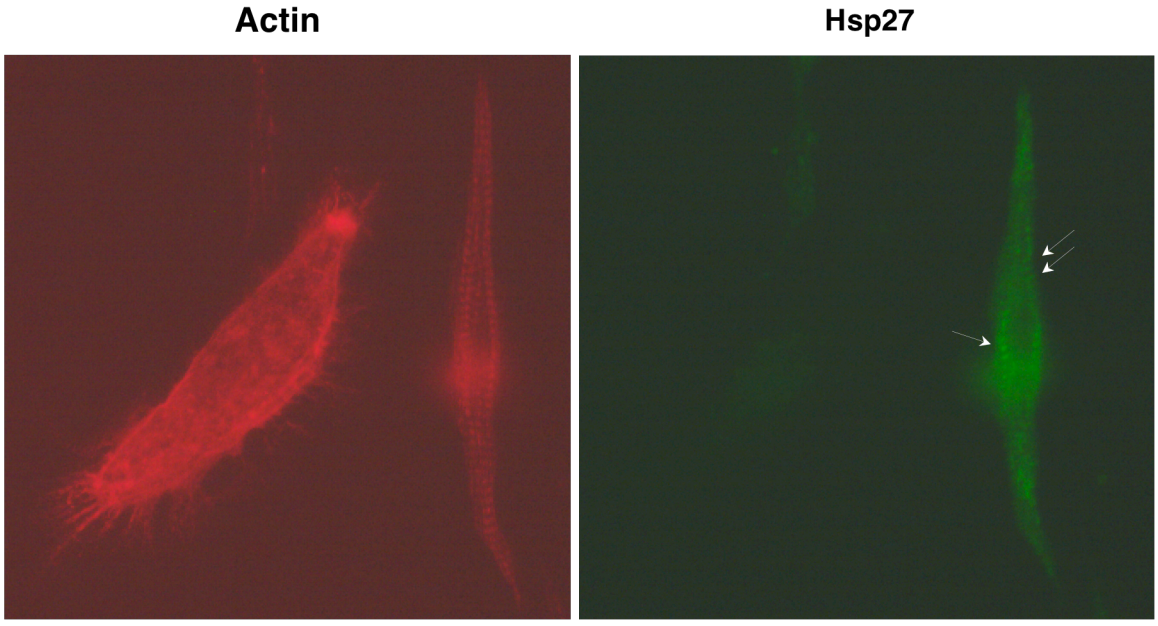


Figure 7: Hsp27 forms sarcomeric-like structure in response to α1-AR stimulation: RNVMs serum starved were incubated for 1 h in the absence or presence of PE. Cells were then fixed, permeabilized, and incubated with anti-Hsp27 polyclonal antibodies as well as FITC conjugated anti-rabbit secondary antibodies and with rhodamine-conjugated-phalloidine to detect the expression of the actin.

5.7 Over-expressing the competitor fragment of AKAP-Lbc 1585-1715 impairs the hypertrophic response of NRVMs induce by α1-ARs stimulation

We have previously shown that AKAP-Lbc mediates α1-ARs induced cardiomyocyte hypertrophy. To determine whether this effect requires the ability of AKAP-Lbc to assemble a p38-activated complex, we determined the impact of disrupting the complex using the competitor fragment (AKAP-Lbc 1585-1715) on the ability of α1-ARs to induce cardiomyocyte hypertrophy. Induction of cardiomyocyte hypertrophy was assessed by measuring cell size as well as sarcomere assembly and reorganization. In RNVMs transfected with GFP alone, treatment with PE for 24 h induced a 70% increase in cell size (Fig.8A).

Interestingly, this effect was impaired in cardiomyocytes expressing AKAP-Lbc-1585-1715-GFP, in which PE treatment could only induce a 40% increase in cell size (Fig. 8B).

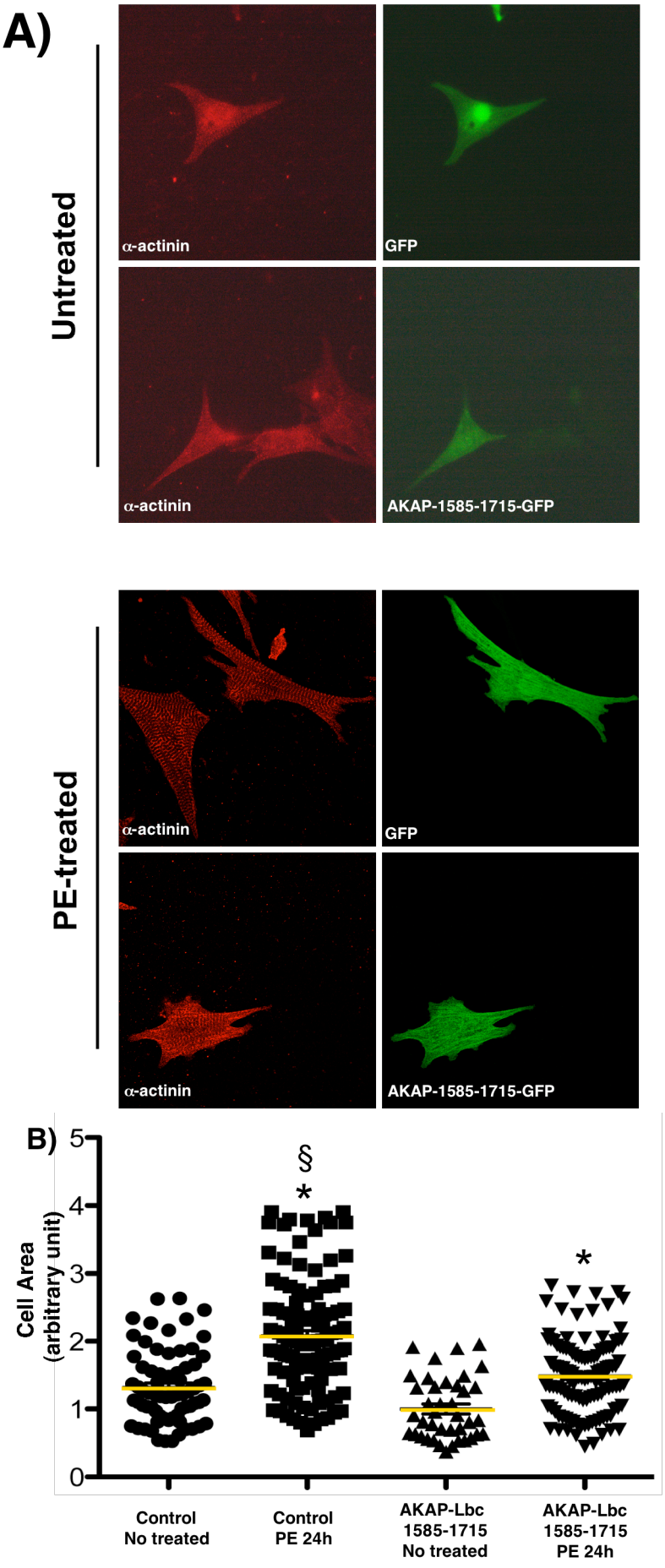


Figure 8: AKAP-Lbc 1585-1715 impairs the hypertrophic response of NRVMs induce by α1-ARs stimulation: RNVMs were transfected with GFP (control) or with GFP-AKAP-Lbc 1585-1715. Seventy-two hours after transfection cardiomyocytes serum starved were incubated for 24 h in the absence or presence of PE. Cells were then fixed, permeabilized, and incubated with anti-α-actinin (red) monoclonal antibodies as well as rhodamine-conjugated anti-mouse secondary antibodies. GFP expression was visualized directly by fluorescent excitation at 490 nm. **(*B*)** Mean cell surface area (± SE) of cardiomyocytes trasfected and treated with or without PE. The cell surface area was determined on a total of 120 GFP-positive cardiomyocytes derived from four independent experiments by using the Image J software. Statistical significance was analyzed by paired Kruskal-Wallis'test. ∗, $P < 0.05$ as compared with the cell surface area measured in PE stimulated cardiomyocytes with control no-stimulated. §, $P < 0.05$ as compared with the cell surface area measured in PE stimulated cardiomyocytes transfeted with GFP-AKAP1585-1715 with control GFP cells.

In agreement with these findings, overexpressing the competitor fragment inhibited the PE induced sarcomere reorganization and assembly.

Taken together these results suggest that the expression of the competitor fragment impairs the hypertrophic response induced by α1-ARs. These finding are in agreement with the hypothesis that AKAP-Lbc/p38 complex promotes cardiomyocyte hypertrophy.

5.8 Over-expressing the longer competitor fragment of AKAP-Lbc 1570-1764 impairs the phosphorylation of Hsp27 induced by α1-ARs stimulation in NRVMs

To provide evidence that the integrity of the AKAP-Lbc/p38 complex is required for the phosphorylation induced by α1-ARs, we determined whether disruption of the AKAP-Lbc complex by overexpressing the competitor fragment encompassing residues 1585-1715 of AKAP-Lbc, could affect PE-induced Hsp27 phosphorylation.

Our results indicate that infected RNVMs with lentiviral vectors encoding the competitor fragment, strongly inhibited the ability of α1-ARs to induce the phosphorylation of Hsp27 (Fig.9 panel 1 lanes 4 and 6). Unfortunately, due to technical problems, it was not possible to detect the fragment in these cardiomyocytes by western blot (data not shown).

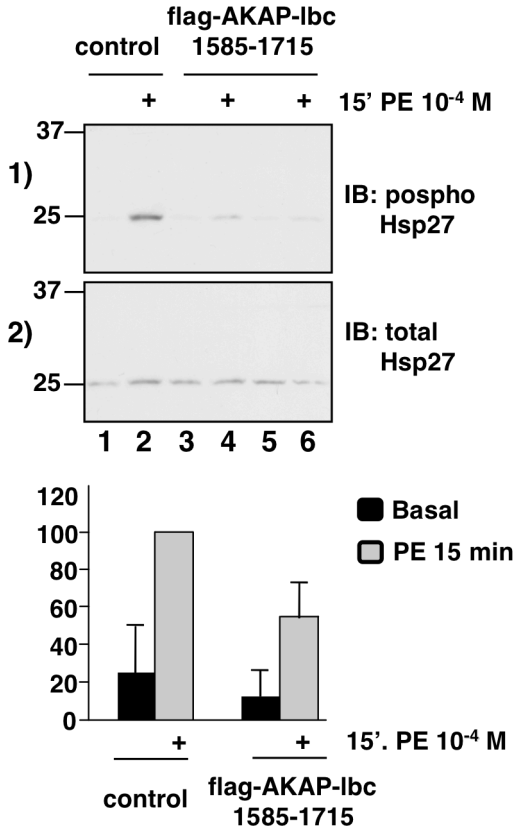


Figure 9 AKAP-Lbc 1570-1764 impairs the phosphorylation of Hsp27 induce by α1-ARs stimulation RNVMs were infected with lentiviruses encoding Flag (control), and Flag-AKAP-Lbc1570-1764 at a moi of 50. Four days after infection, cardiomyocytes serum starved, were incubated 15 minutes with or without (Ctrl) 10^{-4} M phenylephrine (PE). Proteins in the cell lysates were separated by SDS-PAGE and the phosphorylated form of Hsp27, was detected using phospho specific antibody of Hsp27, (upper panel). The amounts of total Hsp27, in the cell lysates were assessed using specific antibodies against Hsp27 (lower panel). Quantitative analysis of phosphorylated Hsp27 was obtained by densitometry. The amount of phospho-Hsp27 was normalized to the total amount of Hsp27. Results are expressed as mean ± S.E. of 8 different experiments.

VII DISCUSSION

In response to various pathological stresses, the heart undergoes a pathological remodeling process that is associated with cardiomyocyte hypertrophy [1-3]. Since cardiac hypertrophy can progress to heart failure, a major cause of lethality worldwide, the intracellular signaling pathways that control cardiomyocyte growth have been subjected of intensive investigation.

While previous studies performed in our laboratory have demonstrated the implication of AKAP-Lbc in the hypertrophic responses activated by α1-ARs and ATIRs, the downstream signaling pathways linking AKAP-Lbc to cardiomyocyte hypertrophy remained to be elucidated [28]. Our findings now indicate that AKAP-Lbc, in RNVMs, is able to organize a macromolecular signaling complex containing RhoA, PKNα, MLTKβ, MKK3β and p38α that participates in the hypertrophic pathway upon α1-ARs activation in primary cultures of RNVMs.

In particular our results indicate that suppression of AKAP-Lbc expression and disruption of the AKAP-Lbc/p38 complex not only reduces PE-induced p38 activation but also inhibits α1-ARs mediated cardiomyocyte hypertrophy as well as sarcomere reorganization.

Interestingly, we could identify the transcriptional factor GATA-4 and the heat shock Hsp27 as effectors that could potentially mediate the hypertrophy and sarcomere reorganization effects of the AKAP-Lbc/p38 complex in cardiomyocytes. Overall these findings identify AKAP-Lbc/p38 complex as a signaling unit involved in the activation of hypertrophic response induced by α1-ARs.

Previous studies have shown that p38 can controls hypertrophy and sarcomere assembly in cultured cardiomyocytes. This conclusion was based on the observation that inhibition of p38 using the specific inhibitor SB203580 or by overexpressing its dominant negative mutant could strongly reduce hypertrophic gene expression as well as sarcomere reorganization upon hypertrophic stimuli such as PE, Ang. II, ET-I [122, 129, 135, 136].

Our current results identify AKAP-Lbc/p38 complex as a mediator of hypertrophic response induced by PE. In fact the disruption of the complex using AKAP-Lbc 1585-1715 competitor fragment can impair hypertrophic signaling responses, indicating that the assembly of PKNα, MLTKβ, MKK3β and p38α by AKAP-Lbc is required for the efficient transduction signals downstream of α1-ARs.

These findings are in agreement with previous results showing that RhoA, PKNα and p38 as mediators of α1-ARs induced cardiomyocyte hypertrophy and sarcomeric reorganization [141]. They also indicate that hypertrophic signals generated by α1-ARs can be integrated at the molecular level by AKAP-Lbc and transmitted via RhoA to p38 activating module.
It is apparent from our results that overexpression of AKAP-Lbc competitor fragment cannot completely inhibit PE induced hypertrophic responses. These results could be explained by an incomplete disruption of the AKAP-Lbc/p38 complex in all cardiomyocytes and/or they indicate that other additional pathways can induce cardiomyocyte hypertrophy.

While our current findings identified a signaling complex involved in the activation of p38 induced by α1-ARs, hypertrophic agonists can promote p38 activation through a variety of different pathways. In fact it has been shown that stimulation of ATIRs promotes p38 activity through a signaling cascade, involving the activation of Rac1, the reactive oxygen species (ROS) and the subsequent activation of the MAPKKK ASK1 and its downstream kinases MKK3/6 [49, 50]. On the other hand, the hypertrophic transforming growth factor (TGF)-beta induces p38 activation via the MAPKKK TAK1 and MKK3/6 [218].
Based on this evidence, it will be interesting to determine whether the transduction elements that composed these pathways are also organized into transduction modules that might specifically activate p38 in response to the stimulation of selected receptors.

The role of p38 in cardiac hypertrophy is still debated. While our current findings are in agreement with previous evidence suggesting that p38α is phosphorylated in RNVMs, we are aware of the fact that it will be crucial to define whether AKAP-Lbc/p38 complex mediates hypertrophic response in vivo.
In this respect, the impact of inhibiting p38 signaling in vivo is matter of controversy. Chronic inhibition of p38 signal by overexpressing dominant negative p38α induces pro-hypertrophic effect suggesting that p38α in vivo is anti-hypertrophic [130, 131].
Moreover surprisingly, cardiac-specific KO mice of p38 did not affect the hypertrophic response induced by the stress. In contrast to these findings, acute activation of p38 signaling in adult mice produces hypertrophy[133].
To understand the role of the AKAP-Lbc/p38 complex in cardiac hypertrophy our group has generated the transgenic mice that specifically overexpress the AKAP-Lbc 1585-1715

competitor fragment in the heart. Expression of the transgene is under control of the myosin heavy chain promoter. Using this strategy we expect to characterize the in vivo role of AKAP-Lbc/p38 complex in cardiac myocytes. Transgenic mice will be subjected to different hypertrophic stimuli (TAC, PE infusion exc.) and the cardiac hypertrophy will be assessed by echocardiography as well as by determining the heart weight index, the activation of hypertrophic genes and by measuring the cardiomyocyte cross-sectional area.

Previous evidence indicates that the transcription factor GATA4 can mediate the transcription of hypertrophic genes downstream of α1-ARs. Activation of GATA4 occurs via a pathway that includes RhoA and p38 [141]. These findings are in line with our current results showing that the AKAP-Lbc/p38 complex mediates α1AR-induced activation of GATA4. α1ARs can promote the expression of GATA4-regulated hypertrophic genes such as ANF, α-skeletal actin, β-Myosin heavy chain exc.
However, these genes are also regulated through the activation of other hypertrophic transcription factors such as MEF2 and SRF. Therefore, to formally demonstrate that AKAP-Lbc/p38 complex mediates cardiomyocyte hypertrophy via GATA4, future experiments will determine whether the silencing of GATA4 can inhibit the hypertrophic responses induced by the overexpression of AKAP-Lbc in RNVMs. In addition to GATA4, p38 is known to activate MEF2. Therefore it will be important to determine whether the AKAP-Lbc/p38 complex also activates MEF2 transcription factor.

In cardiomyocytes Hsp27 plays an important role to stabilize the actin cytoskeleton and the sarcomere in response to stresses.
While it is clear that Hsp27 phosphorylation, through the p38-MK2 pathway, induces its translocation to the actin cytoskeleton and to the sarcomere [162], it is currently unknown whether hypertrophic agonists can promote Hsp27 phosphorylation and translocation, and whether this contributes to their re-organization. Our current results indicate that α1AR stimulation promotes Hsp27 phosphorylation and that the AKAP-Lbc/p38 complex mediates this effect. Interestingly, we could observe the translocation of Hsp27 to the sarcomere and to actin filaments already 1h after PE stimulation. However, we currently do not know whether this effect is required to stabilize actin and sarcomeric structures.
Therefore, future experiments will determine whether Hsp27 silencing in cardiomyocytes can affect actin and sarcomere reorganization induced by α1AR stimulation or by AKAP-

Lbc overexpression. By rescuing Hsp27 expression using either the wild type Hsp27 or its phosphorylation-deficient mutant, we will determine whether the effect of α1-AR and AKAP-Lbc require p38-dependent Hsp27 phosphorylation for sarcomere organization.

In conclusion, the implications of our findings are twofold. Firstly, they have identified the novel hypertrophic signaling complex organized by AKAP-Lbc, (includes RhoA, PKNα, MLTKβ, MKK3β and p38α). This complex mediates activation of p38α downstream of α1AR. Once activated this pathway is able to promote the activation of the hypertrophic transcription factor GATA4. Secondly, they identify a non-genomic pathway activated by this macromolecular signaling complex that leads to the activation of the small chaperone protein Hsp27.

Activation of Hsp27 could play a role in stabilizing actin cytoskeleton and sarcomeres, thus favoring their reorganization in response to hypertrophic agonists.

Overall, this study contributes to our understandings of the role of AKAP-Lbc/p38 complex in cardiomyocyte hypertrophy and in general to the elucidation of the molecular mechanisms controlling cardiac remodeling.

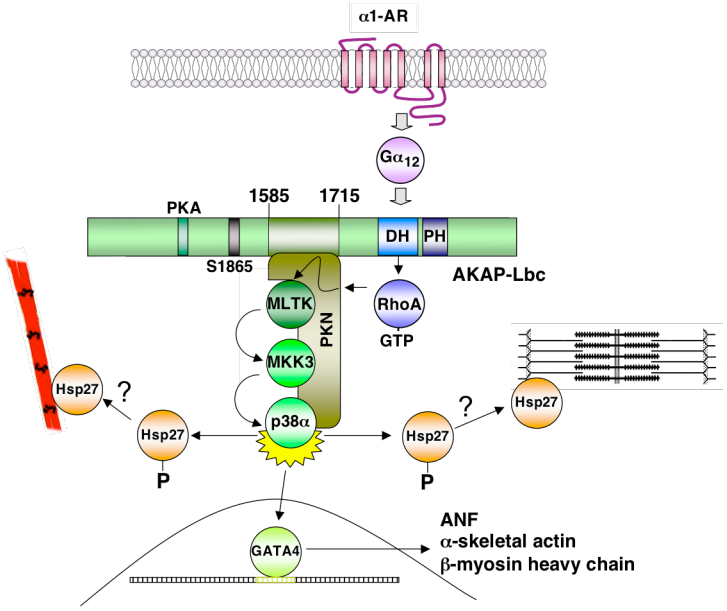


Figure 10. Model for the AKAP-Lbc-p38α activation complex in cardiomyocyte: AKAP-Lbc assembles a signaling complex which includes the scaffolding protein PKNα as well as MLTKβ, MKK3β and p38α. The AKAP-Lbc signaling complex is activated in response to α1-AR stimulation. Activated AKAP-Lbc promotes the formation of RhoA-GTP, which, in turn, induces the activation of a signaling cascade that includes PKNα, MLTKβ, MKK3β and p38α. Once p38 is activated it can regulate the activation of the hypertrophic transcription factor GATA4 and the activation of Hsp27, which is known to protect the cytoskeleton actin and the sarcomere through direct interactions.

VIII. GENERAL CONCLUSIONS

In response to numerous pathologic stimuli, the myocardium undergoes a hypertrophic response characterized by increased myocardial cell size and activation of fetal cardiac genes.

In this context, our earlier work has identified AKAP-Lbc as an anchoring protein expressed in cardiomyocytes that is a key mediator of the hypertrophic responses induced by multiple stimuli that activate G protein-coupled receptors (GPCRs) including catecholamine, angiotensin II (Ang-II) and endothelin 1 (ET-1). However, the pathways whereby AKAP-Lbc promotes hypertrophy were largely unknown.

During my thesis I have investigated the signaling pathways through which the AKAP-Lbc/RhoA complex modulates cardiomyocyte hypertrophy. In particular, in the first part of my work I have identified and characterized a novel signaling complex organized by AKAP-Lbc that promotes p38α activation downstream of α1ARs, while in the second part of my project I have investigated the impact of this complex in cardiomyocyte hypertrophy.

In this context I have identified GATA4 and Hsp27 as potential mediators of the hypertrophic effects of the AKAP-Lbc/p38 complex.

In perspective it will be crucial to characterize the role of the AKAP-Lbc/p38 complex in cardiac remodeling in vivo, as mentioned before, by generating transgenic mice overexpressing the 1585-1715 fragment of AKAP-Lbc in the heart. This will allow us to determine whether disruption of the complex in cardiomyocytes affect the hypertrophic responses induced by various stresses.

Alternatively, it will be important to define the role of AKAP-Lbc/p38 complex in cardiac fibroblasts. In this context, our group has recently shown that AKAP-Lbc is expressed also in cardiac fibroblasts, the most numerous cell type in the heart. In response to hypertrophic stresses cardiac fibroblasts differentiate in cardiac myofibroblasts, which display high proliferation capacity, secrete high amounts of extracellular matrix and promote cardiac fibrosis.

Overall, this study contributes to our understandings of the role of AKAP-Lbc/p38 complex in cardiomyocyte hypertrophy and in general to the elucidation of the molecular mechanisms controlling cardiac remodeling.

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RÉSUMÉ POUR TOUT PUBLIC

L'hypertrophie cardiaque représente un mécanisme d'adaptation du myocarde en réponse à différents stress. Sur le long terme, l'hypertrophie cardiaque peut évoluer vers l'insuffisance cardiaque, l'une des principales causes de morbidité et de mortalité dans les pays industrialisés, pour cette raison, la communauté scientifique est très intéressée à élucider les voies de signalisation qui régulent ce phénomène pathologique dans le cœur.

La protéine kinase A (PKA) est une protéine abondante dans les cellules qui est essentielle pour de nombreux phénomènes cellulaires. Les protéines d'ancrage de la protéine kinase A (AKAPs) constituent une grande famille de protéines qui dirigent la PKA à proximité de ses protéines cibles pour assurer leur régulation. Une nouvelle protéine de cette famille, appelée AKAP-Lbc, a été récemment découverte et fonctionne comme un activateur de RhoA, une protéine impliquée entre autres dans la croissance et la différenciation des cellules cardiaques (cardiomyocytes). Le but général de ce travail de thèse est de comprendre la voie de signalisation activée par le complexe AKAP-Lbc/RhoA au niveau moléculaire et déterminer la fonction dans les cardiomyocytes.

Mes travaux montrent que AKAP-Lbc organise et active un complexe macromoléculaire, comprenant les protéines kinases PKN, MLTK, MKK3 et p38 en réponse à l'activation des récepteurs α1-adrénergiques. Dans les cardiomyocytes, cette voie de signalisation régule le processus de l'hypertrophie par l'activation du facteur de transcription hypertrophique GATA4, et par l'activation de la protéine Hsp27.

Hsp27 joue un rôle crucial dans la protection du cytosquelette des cardiomyocytes. Ce mécanisme est particulièrement important pour la survie des cellules cardiaques au cours du stress hypertrophique.

Pris ensembles, ces études contribuent à comprendre comment le complexe de signalisation formé par AKAP-Lbc régule l'hypertrophie dans les cardiomyocytes. Au-delà de leur intérêt au niveau biochimique, ces travaux pourraient aussi contribuer à la compréhension du phénomène de l'hypertrophie dans le cœur.

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A-Kinase Anchoring Protein (AKAP)-Lbc Anchors a PKN-based Signaling Complex Involved in α_1-Adrenergic Receptor-induced p38 Activation*[S]

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The mitogen-activated protein kinases (MAPKs) pathways are highly organized signaling systems that transduce extracellular signals into a variety of intracellular responses. In this context, it is currently poorly understood how kinases constituting these signaling cascades are assembled and activated in response to receptor stimulation to generate specific cellular responses. Here, we show that AKAP-Lbc, an A-kinase anchoring protein (AKAP) with an intrinsic Rho-specific guanine nucleotide exchange factor activity, is critically involved in the activation of the p38α MAPK downstream of α_{1b}-adrenergic receptors (α_{1b}-ARs). Our results indicate that AKAP-Lbc can assemble a novel transduction complex containing the RhoA effector PKNα, MLTK, MKK3, and p38α, which integrates signals from α_{1b}-ARs to promote RhoA-dependent activation of p38α. In particular, silencing of AKAP-Lbc expression or disrupting the formation of the AKAP-Lbc·p38α signaling complex specifically reduces α_1-AR-mediated p38α activation without affecting receptor-mediated activation of other MAPK pathways. These findings provide a novel mechanistic hypothesis explaining how assembly of macromolecular complexes can specify MAPK signaling downstream of α_1-ARs.

α_1-Adrenergic receptors (α_1-AR)[2] are seven-transmembrane domain receptors coupled to heterotrimeric G proteins of the G_q and G_{12}/G_{13} family (1, 2). Evidence accumulated over the last years indicate that these receptors, besides their well known implication in controlling vascular contractility, glucose metabolism, genitourinary functions, and behavioral responses (3), are also crucially involved in the regulation of various pathological cardiovascular remodeling processes including vascular smooth muscle cell hypertrophy, proliferation, and migration in response to injury (4, 5) as well as cardiac hypertrophy (6–8). It is now evident that mitogen-activated protein kinases (MAPKs) signaling pathways play a central role in mediating many of these pathological responses (1, 9–11).

MAPKs are proline-directed serine/threonine kinases that induce the majority of their physiological effects through phosphorylation and activation of transcription factors and the regulation of the expression of specific sets of genes (12). Mammalian MAPKs can be subdivided into five families including ERK1/2, JNK, p38, ERK3/4, and ERK5, which display different biological functions (12). MAPK signaling cascades are organized into functional signaling modules of three kinases in which a MAP kinase kinase kinase (MAPKKK) phosphorylates and activates a MAP kinase kinase (MAPKK) that, in turn, phosphorylates and activates a MAPK (13). The modular organization of the pathway is controlled by scaffolding proteins that can bind each of the kinases (13). Although the implication of MAPK pathways in the pathophysiological responses induced by α_1-ARs has been extensively studied it is currently unknown how MAPK signaling modules are assembled and activated in response to α1-AR stimulation to generate specific cellular responses.

Several evidences indicates that small molecular weight GTPase RhoA plays a central role in mediating the activation of MAPK pathways downstream of α_1-ARs (1, 10, 14). Rho-GTPases are molecular switches that cycle between an active GTP-bound state, which is able to bind effector proteins, and an inactive GDP-bound state. *In vivo*, the cycling between the GDP- and GTP-bound forms is regulated by guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GDP with GTP. All guanine nucleotide exchange factors exhibiting exchange activity toward Rho GTPases share a Dbl homology domain and an adjacent pleckstrin homology domain (15, 16). The Dbl homology domain is responsible for the guanine nucleotide exchange activity, whereas the pleckstrin homology domain determines the subcellular localization of the exchange factor or contributes to the binding pocket for Rho-GTPases (16).

We identified an exchange factor, termed AKAP-Lbc, which functions as GEF for RhoA as well as an A-kinase anchoring protein (AKAP) (17, 18). Interestingly, AKAP-Lbc is a key mediator of α_1-AR-induced activation of RhoA (2). α_1-ARs enhance AKAP-Lbc Rho-GEF activity through a signaling pathway that requires the α subunit of the heterotrimeric G protein G_{12} (17). On the other hand, AKAP-Lbc inactivation occurs through a mechanism that requires recruitment of the regulatory protein 14-3-3 (19).

Our present results indicate that AKAP-Lbc organizes a p38 MAPK complex composed of RhoA effectors PKNα, MLTK,

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[2] The abbreviations used are: α_1-AR, α_1-adrenergic receptor; GEF, guanine nucleotide exchange factor; AKAP, A-kinase anchoring protein; MLK, mixed lineage kinase.

MKK3, and p38α that specifically promotes RhoA-dependent activation of p38 in response to α1-AR stimulation. They also provide evidence that the ability of AKAP-Lbc to assemble and activate this p38 activation complex is inhibited following the recruitment of the regulatory protein 14-3-3. Overall, these findings provide a novel mechanistic hypothesis explaining how MAPK signaling pathways can be selectively activated by α_1-ARs.

EXPERIMENTAL PROCEDURES

Expression Constructs—AKAP-Lbc fragments encoding amino acids 1585–1922, 1625–1922, 1655–1922, 1715–1922, 1765–1922, 1388–1585, 1388–1655, 1388–1715, and 1388–1765 were PCR-amplified from the AKAP-Lbc pEGFPN1 vector (17) and subcloned at EcoRI/SalI into the pFLAG-CMV6 vector to generate protein fragments fused with the FLAG epitope. A region encoding residues 1388–1922 of AKAP-Lbc was PCR amplified from the AKAP-Lbc pEGFPN1 vector and subcloned at SalI/NotI into pET30a vector to generate protein fragments fused with the histidine tag. The AKAP-Lbc fragment encoding residues 1585–1715 and corresponding to the PKNα binding domain of AKAP-Lbc was PCR amplified from the AKAP-Lbc pEGFPN1 vector and subcloned at EcoRI/SalI into pFLAG-CMV6 and pEGFPN vectors to generate protein fragments fused with the FLAG epitope and GFP, respectively. The FLAG-tagged AKAP-Lbc mutant missing the PKNα binding domain was generated by deleting the region encoding amino acids 1585–1715 by standard PCR-directed mutagenesis using the FLAG-AKAP-Lbc vector (19) as a template.

Double-stranded hairpin (sh) oligonucleotides based upon the human AKAP-Lbc mRNA sequence (GI: 15986728, bases 6688–6706 and 228–246) were cloned into the HindIII and BglII sites in the pSUPER vector. The oligonucleotide sequences used were: human AKAP-Lbc shRNA1 (sense strand), 5′-GTGCGTCTCAATGAGATTT-3′, and human AKAP-Lbc shRNA2 (sense strand), 5′-GGTCAGTTCTGATACATTG-3′.

To generate lentiviral transfer vectors encoding AKAP-Lbc shRNAs, cDNA fragments containing the H1 RNA polymerase III promoter as well as sequences encoding shRNAs were excised using BamHI/SalI from the pSUPER vector and subcloned into the pAB286.1 transfer vector. Mission® lentiviral transfer vectors encoding PKNα shRNAs or a control non-target shRNA were purchased from Sigma. These vectors contain a puromycin cassette that allows the selection of infected cells. The lentiviral packaging vectors pCMVDR8.91 and pMD2. VSVG encode the viral capsid and the vesicular stomatitis virus-G envelope protein, respectively (20).

The full-length cDNA encoding human p38α was PCR amplified from a human heart cDNA library and subcloned at NotI-BamHI into pFLAG-CMV6, BamHI-XhoI into HA-pRK5, or BamHI-Not1 into pGEX4T1 to generate proteins fused to the FLAG and HA epitopes or GST, respectively. Similarly, the full-length cDNA encoding human MLTKβ was PCR amplified from a human heart cDNA library and subcloned at NotI-BamHI into pFLAG-CMV6, BamHI-SalI into HA-pRK5, or BamHI-XhoI into pGEX4T1. Fragments 1–305 and 305–942 of PKNα were amplified from Myc-PKNα (generous gift from Dr. S. Gutkind, NIH, Bethesda, MD) and subcloned at NotI-SalI and NotI/XhoI into pET30a, respectively, to generate fusion proteins with the histidine tag. HA-tagged JNK1, MKK3, MKK6, MEK1, and MEKK1 as well as FLAG-tagged JNK1 constructs were generous gifts from Dr. C. Widmann (Department of Morphology and Cell Biology, University of Lausanne). GFP-ERK1 was obtained from Addgene. Plasmids encoding HA-MLK3 and HA-TAK1 were generous gifts from Dr. L. B. Holzman (University of Michigan Medical School) and Dr. J. Ninomiya-Tsuji (North Carolina State University), respectively. Vectors encoding FLAG-AKAP-Lbc S1565A, AKAP-Lbc S1565A-GFP, and FLAG-tagged AKAP-Lbc fragments encoding residues 1–503, 504–1000, 1001–1387, 1388–1922, 1923–2336, and 2337–2817, as well as 14-3-3β-GFP, were described previously (19).

Expression and Purification of Recombinant Proteins in Bacteria—GST fusion proteins of AKAP-Lbc, p38α, MKK3, and MLTK were expressed using the bacterial expression vector pGEX-4T1 in the BL21(DE3) strain of *Escherichia coli* and purified. Exponentially growing bacterial cultures were incubated 16 h at 16 °C with 1 mM isopropyl 1-thio-β-D-galactopyranoside, and subsequently subjected to centrifugation. Pelleted bacteria were lysed in buffer D (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% (w/v) Triton X-100, 1 μg/ml of aprotinin, 2 μg/ml of leupeptin, 2 μg/ml of pepstatin, and 0.1 mM PMSF), sonicated, and centrifuged at 38,000 × *g* for 30 min at 4 °C. After incubating the supernatants with glutathione-Sepharose beads (GE Healthcare) for 2 h at 4 °C, the resin was washed five times with 10 bed volumes of buffer D and stored at 4 °C.

His_6-tagged fusion proteins of PKNα and AKAP-Lbc were expressed using the bacterial expression vector pET30 in BL21(DE3) bacteria and purified. Bacterial extracts containing His_6-tagged fusion proteins were prepared in buffer E (20 mM Hepes, pH 7.8, 500 mM NaCl, 10 mM imidazole, 1 mM benzamidine, 2 μg/ml of leupeptin, 2 μg/ml of pepstatin). After a 1-min sonication, the lysates were centrifuged at 38,000 × *g* for 30 min at 4 °C. The His_6-tagged fusion proteins were purified by incubating the supernatant with nickel-nitrilotriacetic acid chelating resin (Amersham Biosciences) for 1 h at 4 °C. The resin was then washed 5 times with 10 bed volumes of buffer E and stored at 4 °C. His_6-tagged fusion proteins were eluted from the resin with 20 mM Hepes, pH 7.8, 500 mM NaCl, 300 mM imidazole, 1 mM benzamidine, 2 μg/ml of leupeptin, 2 μg/ml of pepstatin for 1 h at room temperature, dialyzed, and stored at −20 °C. The protein content of the eluates was assessed by Coomassie staining of SDS-PAGE gels.

Production of Lentiviruses—Vesicular stomatitis virus-G (VSVG) pseudotyped lentiviruses were produced by cotransfecting 293-T cells with 20 μg of pAB286.1 vector (21), pAB286.1-AKAP-Lbc shRNA vectors (2), Mission® Non-target shRNA control vector (Sigma) or Mission PKNα shRNA vectors (Sigma), 15 μg of pCMVDR8.91, and 5 μg of pMD2.VSVG (20) using the calcium phosphate method. Culture medium was replaced by serum-free DMEM at 12 h after transfection. Cell supernatants were collected 48 h later, filtered through a 0.45-mm filter unit, and concentrated using Centricon-Plus-70 MW 100,000 columns (Millipore). Virus titers were deter-

mined by infecting 293-T cells using serial dilutions of the viral stocks and by scoring the number of puromycin-resistant clones (at 6 days after infection). Titers determined using these methods were between 5×10^8 and 1.0×10^9 transducing units/ml for viruses generated from pAB286.1 vectors and between 4×10^8 and 8×10^8 transducing units/ml for viruses generated from Mission vectors.

Lentiviral Infection—HEK-293 cells were infected at 60% confluence using pAB286.1-based lentiviruses encoding wild type or mutated AKAP-Lbc shRNAs at a multiplicity of infection of 20 in the presence of 8 μg/ml of Polybrene. Two days after infection puromycin was added to the culture medium at a final concentration of 2 μg/ml. After 4 days of selection, puromycin-resistant cells were collected and amplified in selective medium containing puromycin at a final concentration of 2 μg/ml.

Cell Culture and Transfections—HEK-293 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and gentamycin (100 μg/ml) and transfected at 50–60% confluence in 100-mm dishes using the calcium-phosphate method. For the overexpression of constructs containing the full-length AKAP-Lbc, HEK-293 cells were transfected at 80% confluence in 100- or 35-mm dishes using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, cells were grown for 48 h in DMEM supplemented with 10% fetal calf serum before harvesting. The total amount of transfected DNA was 10–24 μg/100-mm dish and 1–4 μg/35-mm dish.

In Vitro GST Pulldown Experiments—For *in vitro* GST pulldowns, 100 nM bacterially purified His_6-tagged fragments encompassing PKNα residues 1–305 and 305–942 as well as AKAP-Lbc residues 1388–1922 were incubated with glutathione-Sepharose beads (Amersham Biosciences) coupled to GST, or to GST fusion proteins of p38α, MKK3, MLTK, or the AKAP-Lbc fragment encompassing residues 1388–1922 in 0.5 ml of buffer A (20 mM Tris, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, 5 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 1 mM PMSF) for 4 h at 4 °C. The beads were then washed five times with buffer A containing 300 mM NaCl, resuspended in SDS-PAGE sample buffer (65 mM Tris, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, pH 6.8) and boiled for 3 min at 95 °C. Eluted proteins were analyzed by SDS-PAGE and Western blotting.

Immunoprecipitation Experiments—For immunoprecipitation experiments, HEK-293 cells grown in 100-mm dishes and expressing various constructs were lysed in 1 ml of buffer A. Cell lysates were incubated 1 h at 4 °C on a rotating wheel. The solubilized material was centrifuged at 100,000 × *g* for 30 min at 4 °C and the supernatants were incubated 4 h at 4 °C with 20 μl of anti-FLAG M2 affinity resin (Sigma) to immunoprecipitate overexpressed FLAG-tagged proteins. Following a brief centrifugation on a bench-top centrifuge, the pelleted beads were washed five times with buffer C, twice with PBS, and proteins were eluted in SDS-PAGE sample buffer by boiling samples for 3 min at 95 °C. Eluted proteins were analyzed by SDS-PAGE and Western blotting. For immunoprecipitation of endogenous AKAP-Lbc complexes, HEK-293 cells were lysed in 1 ml of buffer B (20 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM PMSF). Soluble proteins were isolated by centrifugation as indicated above and incubated with or without 0.25 mM dithiobis(succinimidyl propionate) for 1 h at 4 °C. Cross-linking reactions were blocked by adding Tris, pH 7.4, to the lysate to a final concentration of 50 mM. Immunoprecipitations were performed as indicated previously (19) by incubating 3 mg of lysate with 4 μg of affinity-purified rabbit polyclonal anti-AKAP-Lbc antibodies (Covance).

p38α, JNK, and MLTK Activity Assays—Transfected HEK-293 cells grown in 100-mm dishes were lysed in 1 ml of buffer C (20 mM Tris, pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM glycerophophate, 5 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 1 mM PMSF). Cell lysates were incubated 10 min at 4 °C on a rotating wheel. The solubilized material was centrifuged at 100,000 × *g* for 30 min at 4 °C. 200 μl of supernatant was incubated either with 2 μl of mouse monoclonal anti-p38α antibodies (Cell Signaling Technology) and 20 μl of protein A-Sepharose beads (Amersham Biosciences) for 2 h at 4 °C to immunoprecipitate endogenous p38α or with 20 μl of anti-FLAG M2 affinity resin (Sigma) for 1 h at 4 °C to immunoprecipitate overexpressed FLAG-tagged JNK1 or MLTK. Following centrifugation on a bench-top centrifuge, the pelleted beads were washed three times with buffer C and twice with a buffer containing 50 mM Tris, pH 7.4, and 5 mM $MgCl_2$. Immunoprecipitates containing p38α or JNK1 were incubated with 1 μg of purified GST-ATF2 (Cell Signaling Technology), whereas those containing FLAG-tagged MLTK were incubated with 1 μg of purified GST-MKK3. Reactions were carried out in 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, and 1 mM ATP-Na_2 for 30 min at 30 °C and ended by the addition of SDS-PAGE sample buffer and loaded on SDS-PAGE gels.

SDS-PAGE and Western Blotting—Samples denatured in SDS-PAGE sample buffer were separated on acrylamide gels and electroblotted onto nitrocellulose membranes. The blots were incubated with primary antibodies and horseradish-conjugated secondary antibodies (Amersham Biosciences) as previously indicated (17). The following affinity purified primary antibodies were used for immunoblotting: affinity purified rabbit polyclonal anti-AKAP-Lbc (Covance, 0.1 mg/ml, 1:1000 dilution), mouse monoclonal anti-FLAG (Sigma, 4.9 mg/ml, 1:2000 dilution), mouse monoclonal anti-GFP (Roche Applied Science, 400 μg/ml, 1:500 dilution), rabbit polyclonal anti-GFP (Roche Applied Science, 400 μg/ml, 1:1000 dilution), rabbit polyclonal anti-HA (Sigma, 1:1000 dilution), mouse monoclonal anti-HA (Sigma, 1:5000 dilution), rabbit polyclonal anti-phospho-p38α (threonine 180 and tyrosine 182) (Cell Signaling Technologies, 1:1000 dilution), rabbit polyclonal and mouse monoclonal anti-p38α (Cell Signaling Technologies, 1:1000 dilution), rabbit polyclonal anti-phospho-ATF2 (threonine 71) (Cell Signaling Technologies, 1:1000 dilution), rabbit polyclonal anti-ATF2 (Cell Signaling Technologies, 1:1000 dilution), mouse monoclonal anti-phospho-MKK3 (serine 189 and threonine 193) (Cell Signaling Technology, 1:500 dilution), mouse monoclonal anti-MKK3 (Assay designs, 1:1000 dilution), mouse monoclonal anti-MLTK (Abnova, 1:500 dilution), rabbit polyclonal anti-MLK3 (Cell Signaling Technology, 1:500 dilution), mouse monoclonal anti-PKNα (BD Biosciences

Pharmingen, 1:1000 dilution), rabbit polyclonal anti-ERK1/2 (Santa Cruz Biotechnology, 1:500 dilution), rabbit polyclonal anti-phospho-ERK1/2 (threonine 202 and tyrosine 204) (Santa Cruz Biotechnology, 1:500 dilution), rabbit polyclonal anti-JNK (Cell Signaling Technologies, 1:500 dilution), rabbit polyclonal anti-phospho-JNK (threonine 183 and tyrosine 185) (Cell Signaling Technologies, 1:500 dilution), mouse monoclonal anti-actin (Sigma, 1:1000 dilution), mouse monoclonal anti-histidine tag (Qiagen 100 μg/ml, 1:1000 dilution).

Statistical Analysis—Statistical significance was analyzed using a Kruskal-Wallis test followed by Mann-Whitney U tests with the Bonferroni corrections.

RESULTS

AKAP-Lbc Mediates α_1-AR-induced p38α MAPK Activation—Evidence collected over the last decade indicates that protein kinases of the MAPK family including ERK, JNK, and p38 play an important role in mediating many of the pathophysiological responses induced by α_1-ARs (1, 9–11). Although several studies indicate that GTPase RhoA plays a central role in mediating activation of MAPK pathways downstream of α_1-ARs (1, 10, 14), it is currently not clear how signals are specifically transferred from activated RhoA to the activation of selected MAPKs.

To initially determine the implication of RhoA in pathways linking α_1-ARs to the activation of ERK1/2, JNK, and p38, HEK-293 cells expressing the HA-tagged α_1-AR were treated for 2 h in the absence or presence of 1 μg/ml of a cell permeable form of the C3 botulism toxin and subsequently incubated with or without 10^{-4} M epinephrine for 10 min. Activation of ERK1/2 and JNK in cell lysates was assessed by Western blot using antibodies recognizing the phosphorylated forms of ERK1/2 and JNK. On the other hand, p38α activity was determined using a kinase assay that measured the ability of immunoprecipitated endogenous p38α to induce the phosphorylation of purified GST-ATF2. Interestingly, inhibition of RhoA impaired by 58 and 69% the ability of α_{1b}-ARs to induce p38α activation under basal conditions and following epinephrine stimulation, respectively (Fig. 1, *A*, *panel 1*, *lanes 7* and *8*, and *B*), without affecting α_1-AR-mediated phosphorylation of ERK1/2 and JNK (supplemental Fig. S1, *A* and *C*, *panel 1*, *lanes 7* and *8*, and *B* and *D*). This suggests that RhoA is involved in the pathway that links α_{1b}-ARs to the activation of p38α.

Based on these results and our previous evidence that the RhoA-specific guanine nucleotide exchange factor AKAP-Lbc mediates RhoA activation downstream of α_{1b}-ARs (2), we raised the question of whether AKAP-Lbc might organize the signaling cascade linking α_{1b}-ARs to the activation of p38α. To address this hypothesis, we initially determined the impact of silencing AKAP-Lbc expression in HEK-293 cells on the ability of α_1-ARs to induce the activation of p38α.

AKAP-Lbc silencing was achieved by infecting cells using lentiviruses encoding two distinct shRNAs directed against a sequence within the Dbl homology domain (shRNA1) and N-terminal regulatory region (shRNA2) of AKAP-Lbc, respectively. Both shRNAs could inhibit AKAP-Lbc expression by about 90% as compared with cells infected with control lentiviruses (Fig. 1*C*, *panel 5*).

Infected cells were transfected with the cDNA encoding the HA-tagged α_{1b}-AR, serum starved for 24 h, and incubated in the absence or presence of epinephrine for 10 min. p38α was then immunoprecipitated and its activity assessed as indicated above.

Silencing of AKAP-Lbc expression significantly reduced the ability of α_{1b}-ARs to induce p38α activation both under basal conditions and following epinephrine stimulation. Basal p38α activation was inhibited between 67 (shRNA1) and 74% (shRNA2), whereas inhibition of epinephrine-induced p38α activation was between 63 (shRNA1) and 72% (shRNA2) (Fig. 1*C*, *panel 1*, *lanes 9–12*, and *D*). Interestingly, re-expression of a silencing resistant mutant of AKAP-Lbc (2) in silenced cells rescued the ability of α_{1b}-AR to promote the activation of p38α (supplemental Fig. S2, *A*, *panel 1*, *lanes 5* and *6*, and *B*).

These results suggest that the inhibition of p38α activation was strictly dependent on reduced AKAP-Lbc expression and not due to an off-target effect. Control experiments revealed that the ability of α_{1b}-ARs to promote phosphorylation of endogenous ERK1/2 and JNK was not affected by AKAP-Lbc silencing (supplemental Fig. 3, *A*, *panels 1* and *3*, *lanes 7* and *8*). These results strongly suggest that AKAP-Lbc specifically contributes to the activation of p38α MAPK induced by α_{1b}-ARs.

To directly determine whether AKAP-Lbc can enhance p38α activation through its ability to activate RhoA, we assessed whether RhoA inhibition could affect the p38α activating potential of the S1565A mutant of AKAP-Lbc, which displays constitutive Rho-GEF activity (19). HEK-293 cells transfected with the FLAG-tagged AKAP-Lbc S1565A mutant were serum starved for 24 h and incubated for 2 h in the absence or presence of 1 μg/ml of C3 botulinum toxin. As shown in Fig. 1*E*, overexpression of the FLAG-tagged AKAP-Lbc S1565A mutant induced a 2.9-fold enhancement of p38α kinase activity, which was reduced after RhoA inhibition (Fig. 1, *E*, *panel 1*, *lanes 2* and *3*, and *F*). Control experiments revealed that this constitutively active AKAP-Lbc mutant was not able to promote phosphorylation of endogenous ERK1/2 and JNK (supplemental Fig. S3, *B* and *C*, *upper panel*, *lane 2*). Altogether, these findings indicate that the AKAP-Lbc·RhoA complex specifically mediates α_1-AR-induced p38α activation.

AKAP-Lbc Interacts with p38α—Our current findings reveal that AKAP-Lbc and RhoA mediate the activation of p38α but not that of ERK1/2 and JNK downstream of α_{1b}-ARs. This suggests the existence of molecular mechanisms that allow the AKAP-Lbc signaling complex to select and activate the p38 effector pathway. One attractive hypothesis would be that AKAP-Lbc and p38α might be maintained within the same macromolecular unit. In this configuration, activating signals could be integrated by AKAP-Lbc and rapidly transmitted to p38α.

To assess whether AKAP-Lbc could form a complex with p38, we determined whether HA-tagged p38α could be co-immunoprecipitated with FLAG-tagged AKAP-Lbc from lysates of transfected HEK-293 cells. As shown in Fig. 2*A*, anti-FLAG antibodies could immunoprecipitate HA-p38α from cells expressing the FLAG-tagged AKAP-Lbc, but not from cells transfected with the empty pFLAG vector (Fig. 2*A*, *upper panel*, *lanes 1* and *2*). Using a similar approach we could show that

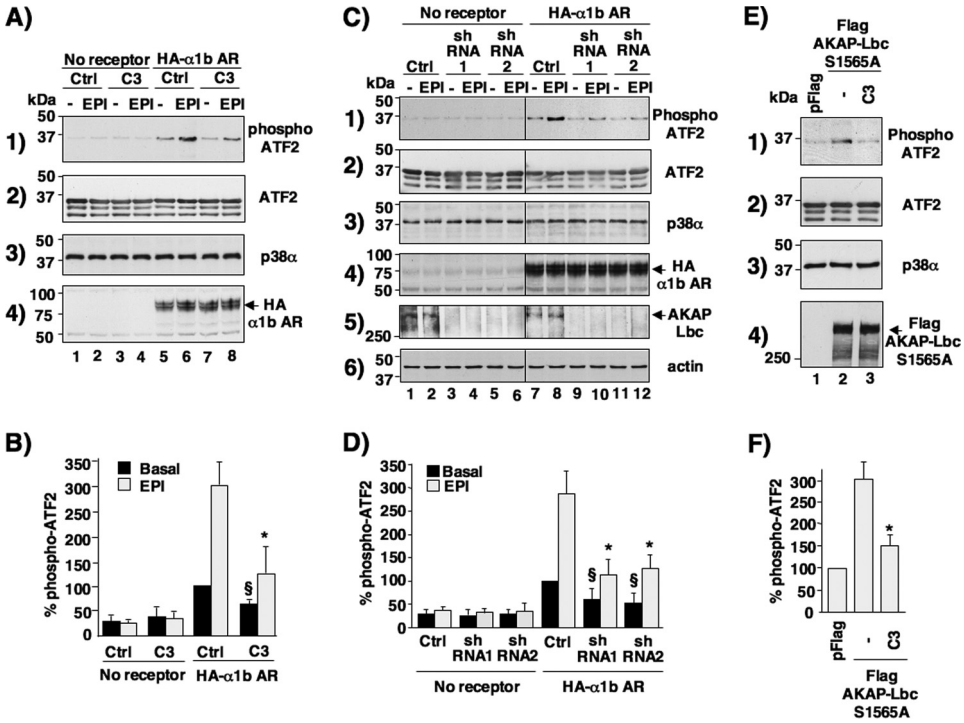

FIGURE 1. **AKAP-Lbc mediates α_1-AR-induced p38α activation.** *A*, HEK-293 cells were transfected with the empty pRK5 plasmid or the cDNA encoding the HA-tagged α_{1b}-AR. After a 24-h serum starvation, cells were incubated for 2 h with or without 1 μg/ml of purified C3 toxin and incubated for 15 min with or without (*Ctrl*) 10^{-4} M epinephrine (*EPI*). Cell lysates where subjected to immunoprecipitation using anti-p38α monoclonal antibodies. Kinase reactions were performed by incubating p38α immunoprecipitates with 1 μg of purified GST-ATF2 and in the presence of ATP. Phospho-GST-ATF2 was detected by immunoblot using rabbit polyclonal antibodies recognizing phosphothreonine 71 of ATF2 (*panel 1*). The amounts of GST-ATF2, p38α, and HA-α1b-ARs were assessed using polyclonal antibodies against ATF2 (*panel 2*), p38α (*panel 3*), and the HA epitope (*panel 4*), respectively. *B*, quantitative analysis of phosphorylated ATF2 was obtained by densitometry. The amount of phospho-ATF2 was normalized to the total amount of ATF2 and p38α. Results are expressed as mean ± S.E. of 3 different experiments. §, $p < 0.05$ as compared with phospho-ATF2 levels measured in untreated control cells expressing HA-α_{1b}-ARs. *, $p < 0.05$ as compared with phospho-ATF2 levels measured in epinephrine-treated control cells expressing HA-α_{1b}-ARs. *C*, HEK-293 cells infected with control lentiviruses or lentiviruses encoding AKAP-Lbc shRNAs were transfected with the empty pRK5 plasmid or the cDNA encoding the HA-tagged α_{1b}-AR. After a 24-h serum starvation, cells were incubated for 15 min with or without (*Ctrl*) 10^{-4} M epinephrine, lysed, and subjected to immunoprecipitation using monoclonal anti-p38α antibodies. Kinase reactions and detection of phospho-ATF2 (*panel 1*), ATF2 (*panel 2*), p38α (*panel 3*), and HA-α_{1b}-ARs (*panel 4*) were performed as indicated in *A*. Expression of endogenous AKAP-Lbc and actin was detected using affinity purified anti-AKAP-Lbc polyclonal antibodies (*panel 5*) and anti-actin monoclonal antibodies (*panel 6*). *D*, quantitative analysis of phospho-ATF2 was obtained by densitometry as indicated in *B*. §, $p < 0.05$ as compared with phospho-ATF2 levels measured in untreated control cells expressing HA-α_{1b}-ARs. *, $p < 0.05$ as compared with phospho-ATF2 levels measured in epinephrine-treated control cells expressing HA-α_{1b}-ARs. *E*, HEK-293 cells were transfected with cDNA encoding the FLAG-tagged S1565A mutant of AKAP-Lbc. After a 24-h serum starvation, cells were incubated for 2 h with or without 1 μg/ml of purified C3 toxin. Kinase activity of immunoprecipitated p38α and detection of phospho-ATF2 (*panel 1*), ATF2 (*panel 2*), and p38α (*panel 3*) in cell lysates was determined as indicated in *A*. Expression of the FLAG-tagged AKAP-Lbc S1565A mutant was assessed using monoclonal antibodies against the FLAG tag (*panel 4*). *F*, quantitative analysis of phosphorylated ATF2 was obtained by densitometry as indicated in *B*. *, $p < 0.05$ as compared with phospho-ATF2 levels measured in untreated cells expressing FLAG-AKAP-Lbc S1565A.

HA-JNK1 and GFP-ERK1 do not form stable complexes with AKAP-Lbc vector (Fig. 2, *B* and *C*, *upper panel*, *lanes 1* and *2*). These results suggest that AKAP-Lbc specifically binds and activates p38α. The p38 family of MAPK is constituted by four members (p38α, p38β, p38γ, and p38δ) (22). Co-immunoprecipitation experiments performed using the different recombinant p38 kinases indicate that FLAG-AKAP-Lbc interacts mainly with p38α, to a lesser extent with p38β, but not with p38γ and p38δ (results not shown).

AKAP-Lbc Assembles a p38α Activation Module—MAPKs are activated by protein kinase cascades in which the MAPK is phosphorylated and activated by a MAP kinase kinase (MAPKK) that is, in turn, phosphorylated and activated by a MAP kinase kinase kinase (MAPKKK). It is well established that p38α can be activated by the MAPKKs MKK3, MKK4, and MKK6 (23). In turn, these three p38-activating kinases can be phosphorylated and activated by several MAPKKKs such as TAK1, members of the mixed lineage kinase (MLK) family including MLK3, MLTK, and DLK, and several members of the MEKK family of protein kinases (23, 24). Because these kinases have been described to be organized into signaling complexes to create functional MAPK modules, we have investigated the possibility that kinases known to act upstream of p38α would also associate with AKAP-Lbc.

To address this point, we performed coimmunoprecipitation experiments from HEK-293 cells that were transiently transfected with the cDNA encoding the HA-tagged forms of the MAPKKs MKK3, MKK4, and MKK6 (Fig. 2*D*), as well as of the MAPKKKs MLK3, MEK1, TAK1 (Fig. 2*E*), and MLTK (Fig. 2*F*) in combination with the empty FLAG vector (pFLAG) or the FLAG-tagged AKAP-Lbc. After immunoprecipitating the anchoring protein using anti-FLAG antibodies, anti-HA antibodies were used to immunoblot the immunoprecipitated samples. Western blots revealed that MKK3 (Fig. 2*D*, *upper panel*, *lane 5*) as well as kinases belonging to MLK family including MLK3 and MLTK (Fig. 2, *E*, *upper panel*, *lane 4,* and *F, upper panel*, *lane 2*) could specifically co-immunoprecipitate with AKAP-Lbc, whereas the MAPKKs MKK4 and MKK6 as well as

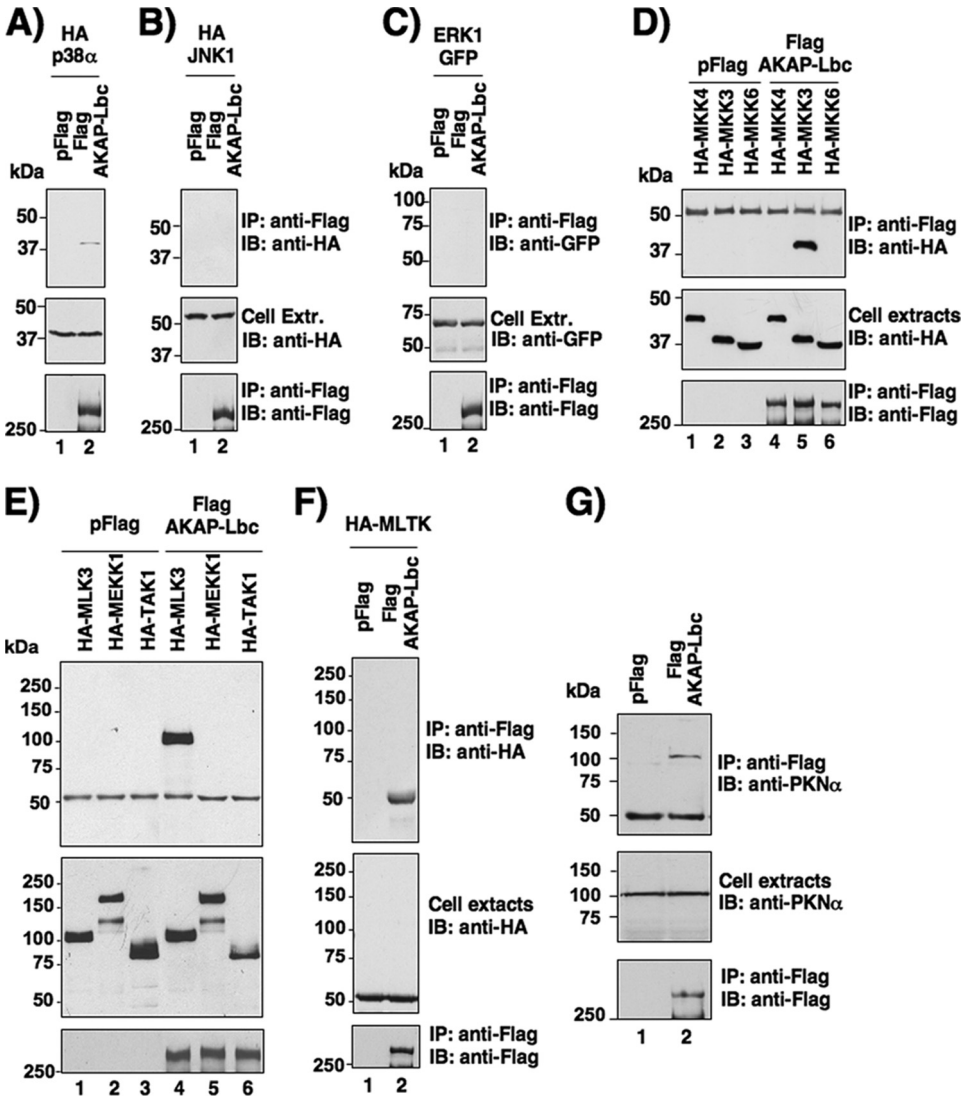

FIGURE 2. **AKAP-Lbc assembles a p38α activation complex.** *A–C*, extracts from HEK-293 cells transfected with the plasmids encoding HA-tagged p38α (*A*), HA-tagged JNK1 (*B*), or GFP-tagged ERK1 (*C*) in combination with the empty FLAG vector (*lane 1*) or the vector encoding FLAG-tagged AKAP-Lbc (*lane 2*) were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using anti-HA monoclonal antibodies to detect HA-p38α and HA-JNK1 (*A* and *B*, *upper* and *middle panels*), anti-GFP polyclonal antibodies to detect GFP-ERK1 (*C*, *upper* and *middle panels*), or anti-FLAG monoclonal antibodies to detect FLAG-AKAP-Lbc (*lower panels*). *D*, HEK-293 cells were transfected with the plasmids encoding HA-tagged MKK4, MKK3, or MKK6 in combination with the vector encoding FLAG-tagged AKAP-Lbc. Immunoprecipitations and Western blots were performed as indicated in *A*. *E* and *F*, HEK-293 cells were transfected with the plasmids encoding HA-tagged MLK3, MEKK1, TAK1 (*E*) or MLTK (*F*), in combination with the empty FLAG vector or the plasmid encoding FLAG-tagged AKAP-Lbc. Immunoprecipitations and Western blots were performed as indicated in *A*. *G*, extracts from HEK-293 cells transfected with the empty FLAG vector or the plasmid encoding FLAG-tagged AKAP-Lbc were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using anti-PKNα monoclonal antibodies. Results are representative of at least three independent experiments.

the MAPKKKs MEKK1 and TAK1 did not (Fig. 2, *D*, *upper panel*, *lanes 4* and *6*, and *E*, *upper panel*, *lanes 5* and *6*). These results suggest that AKAP-Lbc can interact with a signaling module composed of p38α, its upstream kinase MKK3, and MAPKKKs of the MLK family such as MLK3 or MLTK.

However, MLKs have never been reported as direct effectors of RhoA. Accordingly, control experiments performed in our laboratory using purified kinases failed to detect MLK activation by RhoA (results not shown). This raises the question of how the AKAP-Lbc·RhoA complex can transmit signals to the MLK-MKK3-p38α module. In this context, previous evidence indicates that protein kinase Nα (PKNα), a well characterized effector of RhoA, can act as an upstream activating kinase of MLTK (25). Based on these findings, we raised the hypothesis of whether PKNα could also bind to AKAP-Lbc. Interestingly, Western blots performed on FLAG-AKAP-Lbc immunoprecipitates indicate that endogenous PKNα can co-immunoprecipitate with the anchoring protein (Fig. 2*G*, *upper panel*, *lane 2*).

In a similar set of experiments, we could show that p38α, MKK3, MLTK, and PKNα endogenously expressed in HEK293 cells could form a complex with endogenous AKAP-Lbc. This is shown by the fact that p38α, MKK3, MLTK, and PKNα could be detected in AKAP-Lbc immunoprecipitates (Fig. 3, *A*, *upper* and *middle panels*, *lane 3*, and *B*, *upper* and *middle panels*, *lane 3*). On the other hand, no interaction between endogenous

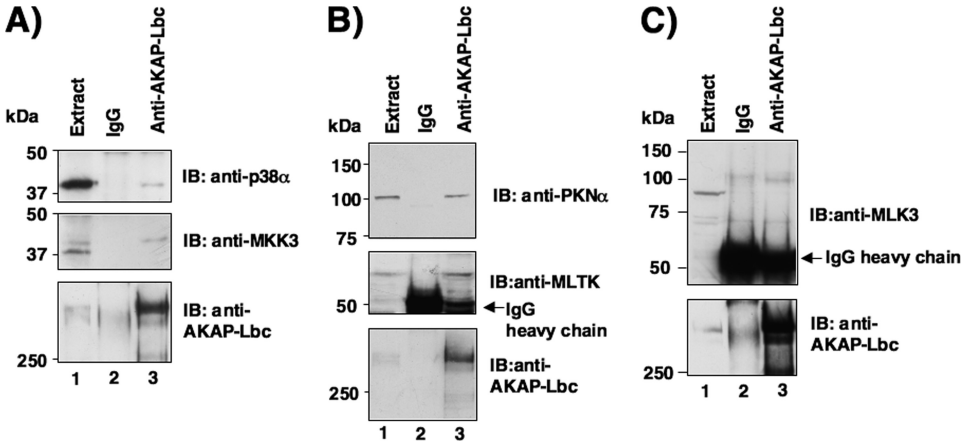

FIGURE 3. **AKAP-Lbc interacts with endogenous p38α, MKK3, MLTK, and PKNα.** HEK-293 cell extracts were subjected to immunoprecipitation with either non-immune IgGs or affinity purified anti-AKAP-Lbc polyclonal antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using anti-p38α (*A*, *upper panel*), anti-MKK3 (*A*, *middle panel*), anti-PKNα (*B*, *upper panel*), anti-MLTK (*B*, *middle panel*), anti-MLK3 (*C*, *upper panel*), or affinity purified anti-AKAP-Lbc polyclonal antibodies (*lower panels*). Results are representative of at least three independent experiments.

AKAP-Lbc and MLK3 could be detected (Fig. 3*C*, *upper panel*, *lane 3*). Therefore, whereas overexpressed AKAP-Lbc has the potential of binding both MLK3 and MLTK (Fig. 2, *E* and *F*), endogenous AKAP-Lbc seems to preferentially assemble a complex that specifically contains MLTK. Collectively these results suggest that AKAP-Lbc can assemble a large signaling complex containing PKNα, MLTK, MKK3, and p38α that can link RhoA activation to the stimulation of the p38 transduction pathway.

Mapping of the Kinase Binding Sites on AKAP-Lbc—To identify the binding site for p38α as well as its upstream activating kinases on AKAP-Lbc, we initially generated a series of FLAG-tagged AKAP-Lbc fragments encompassing residues 1–503, 504–1000, 1001–1387, 1388–1922, 1923–2336, and 2337–2817 (supplemental Fig. S4*A*). The fragments were initially expressed in HEK-293 cells in combination with HA-MKK3 and interactions assessed by co-immunoprecipitation. The FLAG-tagged fragments were immunoprecipitated from cell lysates using anti-FLAG antibodies and the presence of associated kinases detected using anti-HA antibodies. Our results indicate that MKK3 interact exclusively with the fragment of AKAP-Lbc included between residues 1388 and 1922 (supplemental Fig. S4, *upper panel*, *lane 5*).

We have subsequently further narrowed the binding site using shorter FLAG-tagged AKAP-Lbc fragments derived from the 1388–1922 region (Fig. 4*A*). As shown in Fig. 4*B*, our results indicate that HA-MKK3 interacts with AKAP-Lbc in a minimal region encompassing residues 1585–1715 (Fig. 4*B*, *upper panel*).

To validate these findings and assess whether the identified domain was also required for binding the other kinases, we determined the impact of deleting residues 1585–1715 from the AKAP-Lbc on its ability to associate with HA-p38α, HA-MKK3, HA-MLTK, and endogenous PKNα in co-immunoprecipitation experiments (Fig. 4, *C–F*). The deletion reduced significantly the ability of all the tested kinases to co-immunoprecipitate with the FLAG-tagged AKAP-Lbc (Fig. 4, *C–F*, *upper panel*, *second* and *third lanes*). These findings suggest that residues 1585–1715 form a binding site that recruits p38α as well as its upstream activating kinases MKK3, MLTK, and PKNα.

PKNα Directly Binds AKAP-Lbc, p38α, MKK3, and MLTK—Although our current results suggest that AKAP-Lbc interacts with PKNα, MLTK, MKK3, and p38α, they do not indicate how the complex is organized. Based on previous findings showing that PKNα can act both as an upstream activating kinase of MLTK and as a scaffolding protein (25) we hypothesized that it could recruit p38α, MKK3, and MLTK to AKAP-Lbc. Therefore, we determined whether PKNα could directly associate with AKAP-Lbc as well as with the other kinases.

To assess whether the interaction of p38α, MKK3, and MLTK with AKAP-Lbc occurs through a direct interaction or whether it is mediated through PKNα, we monitored the ability of purified GST fusion proteins of p38α, MKK3, and MLTK, and AKAP-Lbc fragment 1388–1922 to associate with purified His_6-tagged N-terminal or C-terminal fragments of PKNα (His-PKN-(1–305) and His-PKN-(305–942), respectively), or with the His_6-tagged 1388–1922 fragment of AKAP-Lbc using an *in vitro* pulldown assay. Interestingly, our results indicate that p38α, MKK3, and MLTK, as well as AKAP-Lbc can directly bind the C-terminal but not the N-terminal fragment of PKNα (Fig. 5, *A*, *upper panel*, *lanes 3*, *5*, *7*, and *9*, *B*, *upper panel*, *lanes 2–6*). A weak direct interaction could also be detected between AKAP-Lbc and MKK3 (Fig. 5*A*, *upper panel*, *lane 6*), whereas no binding was observed between AKAP-Lbc and p38α or MLTK (Fig. 5*A*, *upper panel*, *lanes 4* and *8*). These results indicate that AKAP-Lbc can directly bind PKNα, which, in turn can recruit p38α, MKK3, and MLTK. The association between AKAP-Lbc and MKK3 could stabilize the formation of the complex.

PKNα Is Required for Proper Assembly of the AKAP-Lbc·p38α Signaling Complex—To assess whether PKNα could contribute to assembly of the AKAP-Lbc·p38α complex inside cells, we determined the impact of silencing PKNα expression in HEK-293 cells on the interaction of AKAP-Lbc with p38α and MKK3.

PKNα silencing was achieved by infecting cells using lentiviruses encoding shRNAs directed against PKNα. Using this approach, PKNα expression could be inhibited by 80–90% as

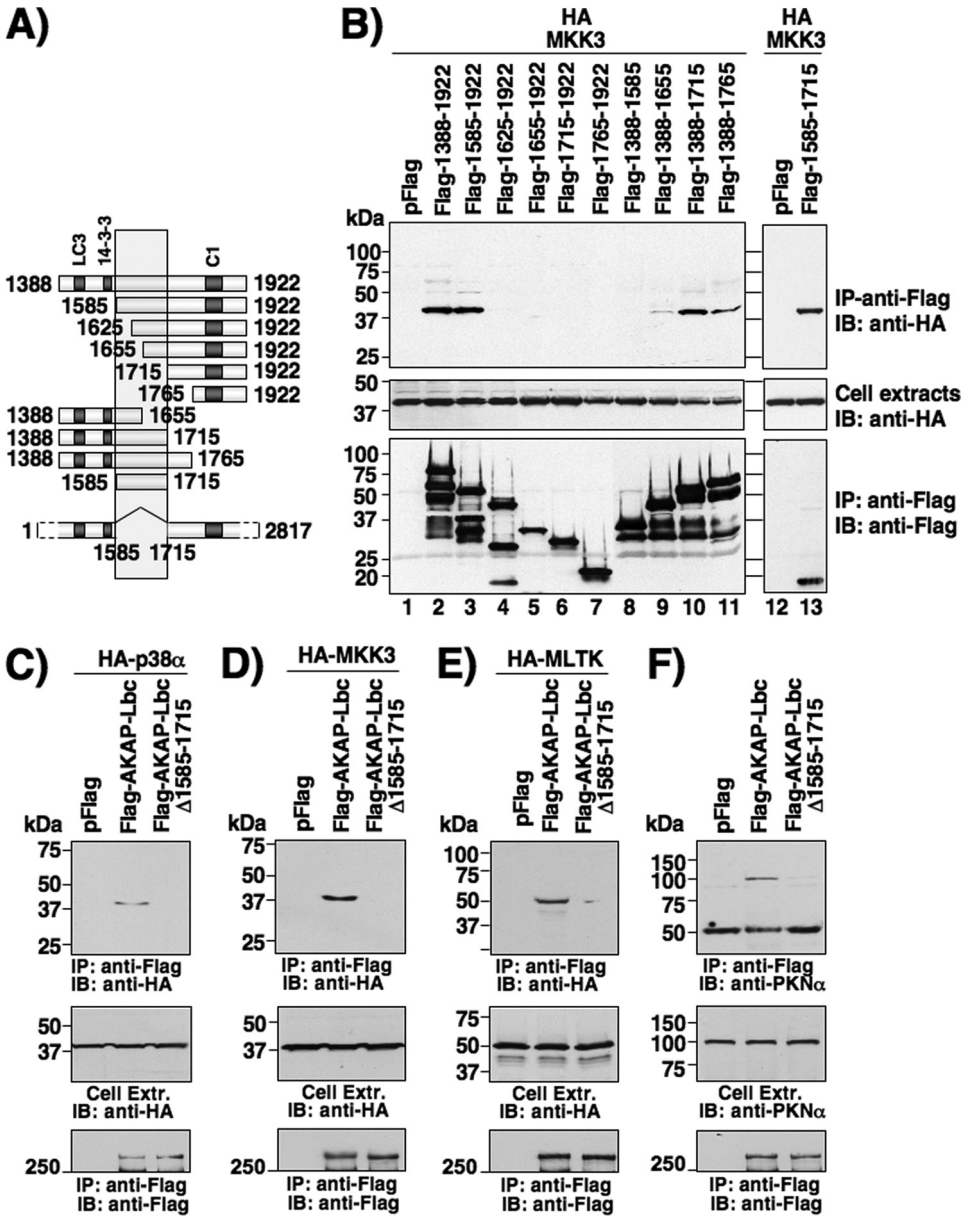

FIGURE 4. **Mapping the kinase interaction sites on AKAP-Lbc.** *A*, schematic representation of the AKAP-Lbc fragments used for the mapping experiments. The minimal binding site (residues 1585–1715) is *boxed*. LC3 and 14-3-3 binding sites as well as the C1 region (*C1*) are shown. *B*, HEK-293 cells were transfected with HA-tagged MKK3 in combination with either the empty FLAG vector or FLAG-tagged fragments of AKAP-Lbc indicated in *A*. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates and cell extracts were revealed using anti-HA polyclonal antibodies to detect the HA-tagged MKK3 (*upper* and *middle panels*), or anti-FLAG monoclonal antibodies to detect the FLAG-tagged AKAP-Lbc fragments (*lower panel*). *C–F*, extracts from HEK-293 cells transfected with plasmids encoding the empty FLAG vector, FLAG-AKAP-Lbc, or the FLAG-AKAP-Lbc Δ1585–1715 mutant in combination with the vectors encoding HA-tagged p38α (*C*), MKK3 (*D*), MLTK (*E*), or the empty pRK5 vector (*F*). Western blots of the immunoprecipitates (*IP*) and the cell extracts were revealed using anti-HA polyclonal antibodies to detect HA-tagged p38α, MKK3, and MLTK (*C–E*, *upper* and *middle panels*), anti-PKNα monoclonal antibodies to detect endogenous PKNα (*F*, *upper* and *middle panels*), or anti-FLAG monoclonal antibodies to detect FLAG-AKAP-Lbc (*lower panels*).

compared with cells infected with control lentiviruses (Fig. 6*A*, *panel 6*).

AKAP-Lbc was immunoprecipitated from infected cells using affinity purified anti-AKAP-Lbc antibodies and the presence of associated p38α and MKK3 revealed by Western blot. As shown in Fig. 6*A*, silencing of PKNα expression significantly reduced the amount of p38α and MKK3 interacting with endogenous AKAP-Lbc (Fig. 6*A*, *panels 1* and *3*, *lane 4*). This suggests that PKNα favors the association of p38α and MKK3 with AKAP-Lbc.

PKNα Mediates AKAP-Lbc-induced Activation of MLTK—Previous findings have shown that PKNα can directly phosphorylate and activate MLTK (25). Based on this evidence, we determined whether PKNα was required to transmit activating signals from AKAP-Lbc to MLTK. To address this point, we assessed whether silencing of PKNα using shRNAs targeting two distinct regions of the kinase (PKN shRNA1 and PKN shRNA2) could affect the ability of the S1565A mutant of AKAP-Lbc, which displays constitutively Rho-GEF activity, to activate MLTK.

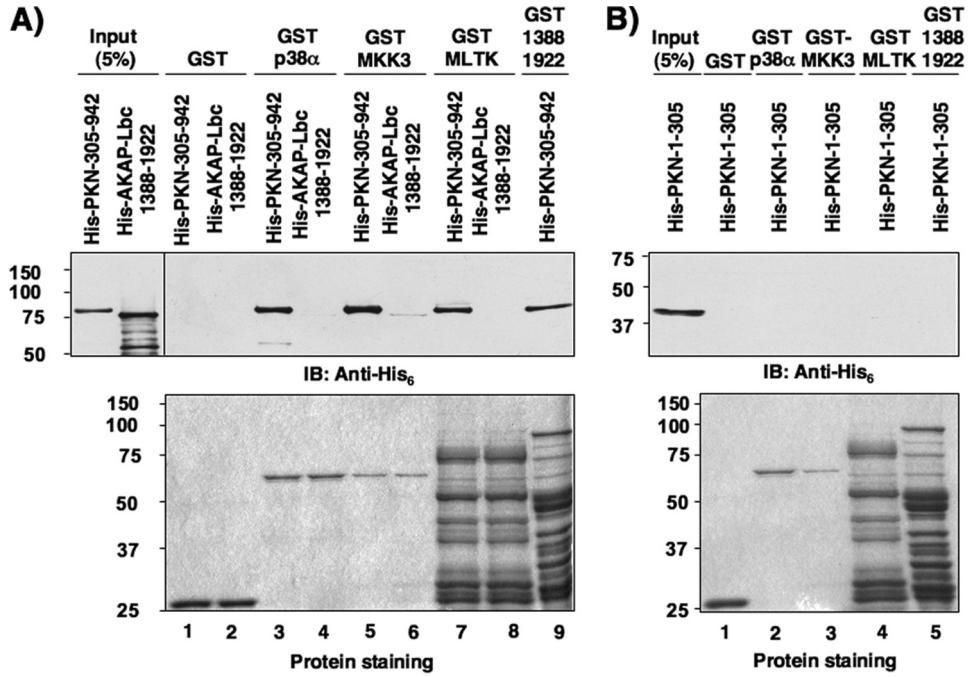

FIGURE 5. **PKNα directly interacts with MLTK, MKK3, p38α, and AKAP-Lbc.** *A*, bacterially purified His_6-tagged fragments (100 nM) encompassing residues 305–942 of PKNα and 1388–1922 of AKAP-Lbc were incubated with glutathione-Sepharose beads coupled to 2 μg of GST alone, or GST-tagged p38α, MKK3, MLTK, and AKAP-Lbc-(1388–1922). Associated His_6-tagged fragments were detected using anti-His_6 monoclonal antibodies (*upper panel*). A control protein staining indicating the expression level of the different GST-tagged constructs used in the pulldown assay is shown (*lower panel*). *B*, bacterially purified His_6-tagged fragments encompassing residues 1–305 of PKNα (100 nM) were incubated with glutathione-Sepharose beads coupled to 2 μg of GST alone, or GST-tagged p38α, MKK3, MLTK, and AKAP-Lbc 1388–1922. Associated His_6-tagged fragments were detected as indicated in *A*.

Infected cells were transfected with the cDNAs encoding FLAG-MLTK and the GFP-tagged AKAP-Lbc S1565A mutant. After a 24-h serum starvation, FLAG-MLTK was then immunoprecipitated and its ability to phosphorylate purified GST-MKK3 determined using an *in vitro* kinase assay. Interestingly, silencing of PKNα expression significantly reduced the ability of AKAP-Lbc S1565A to promote MLTK activation (Fig. 6*B*, *panel 1*, *lanes 5* and *6*) suggesting that PKNα is required for AKAP-Lbc-mediated MLTK activity.

Disruption of AKAP-Lbc Complex Impairs α_1-AR-mediated p38α Activation—Based on the mapping studies presented above, we investigated the possibility of whether a fragment of AKAP-Lbc encompassing residues 1585–1715 could be used as a competitor to disrupt the endogenous complexes formed by AKAP-Lbc and the various kinases. Such a competitor fragment could represent a valuable tool to study the role of the AKAP-Lbc signaling complex in the activation of p38α inside cells.

A GFP fusion of the competitor fragment was expressed in HEK-293 cells and its ability to inhibit the binding of HA-MKK3, HA-MLTK, as well as PKNα to the FLAG-tagged fragment of AKAP-Lbc encompassing residues 1388–1922 was assessed by co-immunoprecipitation (supplemental Fig. S5). Interestingly, overexpression of the fragment reduced the interaction between AKAP-Lbc and the various kinases by more than 80% suggesting that it can act as an efficient competitive inhibitor (supplemental Fig. S5, *A–C*, *upper panel*, *lane 3*). Based on these results we determined the impact of overexpressing the GFP-tagged competitor fragment on the ability of α_{1b}-adrenergic receptors to induce p38α activation in HEK-293 cells.

Interestingly, expression of the competitor fragment reduced by 62 and 58% the ability of α_{1b}-ARs to promote p38α activation under basal conditions and in response to epinephrine stimulation, respectively (Fig. 7, *A*, *panel 1*, *lanes 7* and *8*, and *B*), without affecting receptor-induced phosphorylation of ERK1/2 and JNK1 (supplemental Fig. S6, *A* and *B*, *panel 1*, *lanes 3* and *4*). These findings suggest that the integrity of the complex formed by AKAP-Lbc and the various kinases is required for the activation of p38α induced by α_{1b}-ARs.

Binding of 14-3-33 to AKAP-Lbc Inhibits the Recruitment of PKNα and Reduces p38α Activation—We previously demonstrated that recruitment of the regulatory protein 14-3-3 to a motif located at position 1565 within the N-terminal regulatory region of AKAP-Lbc strongly inhibits the Rho-GEF activity of the anchoring protein (19). Our current results indicate that this site is located in close proximity of the binding domain for the p38α activation complex. This raises the possibility that 14-3-3 recruitment could also interfere with the interaction of AKAP-Lbc with the p38α signaling complex and therefore inhibit AKAP-Lbc-mediated p38 activation.

To address this question, we initially determined whether overexpression of 14-3-3β in HEK-293 cells could affect the ability of AKAP-Lbc to associate with PKNα. HEK-293 cells were transfected with FLAG AKAP-Lbc together with increasing amounts of GFP-tagged 14-3-3β. After immunoprecipitating the anchoring protein using anti-FLAG antibodies, anti-PKNα antibodies were used to immunoblot the immunoprecipitated samples. Western blots revealed that overexpression of increasing amounts of 14-3-3β progressively reduced the ability of PKNα to co-immunoprecipitate with AKAP-Lbc (Fig. 8, *A*, *panel 1*, *lanes 2–4*, and *B*). In line with these results,

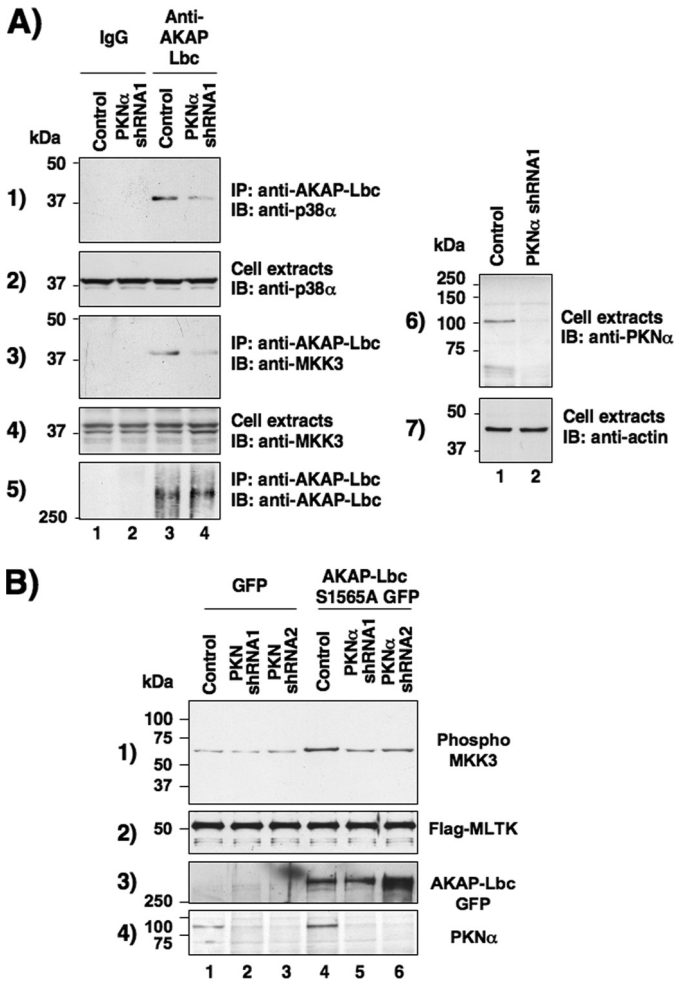

FIGURE 6. **Silencing of PKNα inhibits AKAP-Lbc-mediated activation of MLTK.** *A*, extracts from HEK-293 cells infected with control lentiviruses (*control*) or lentiviruses encoding PKNα shRNAs (*PKNα shRNA1*) were subjected to immunoprecipitation (*IP*) with either non-immune IgGs or affinity purified anti-AKAP-Lbc polyclonal antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed using antibodies against p38α (*panels 1* and *2*), MKK3 (*panels 3* and *4*), AKAP-Lbc (*panel 5*), PKNα (*panel 6*), or actin (*panel 7*). *B*, HEK-293 cells infected with control lentiviruses or lentiviruses encoding PKNα shRNAs (*shRNA1* and *shRNA2*) were transfected with the plasmids encoding FLAG-MLTK in the presence of the vector encoding GFP or the GFP-tagged AKAP-Lbc S1565A mutant. After a 24-h serum starvation, cells were lysed and FLAG-MLTK was subjected to immunoprecipitation using anti-FLAG monoclonal antibodies. Kinase reactions were performed by incubating FLAG-MLTK immunoprecipitates with 1 μg of purified GST-MKK3 and in the presence of ATP. Phospho-GST-MKK3 was detected by immunoblot (*IB*) using a rabbit polyclonal antibody recognizing phosphoserine 189 and phosphothreonine 193 of MKK3 (*panel 1*). The amounts of immunoprecipitated FLAG-MLTK as well as the expression of AKAP-Lbc S1565A-GFP and PKNα in cell lysates were assessed using antibodies against the FLAG tag (*panel 2*), GFP (*panel 3*), and PKNα (*panel 4*), respectively. Results are representative of three independent experiments.

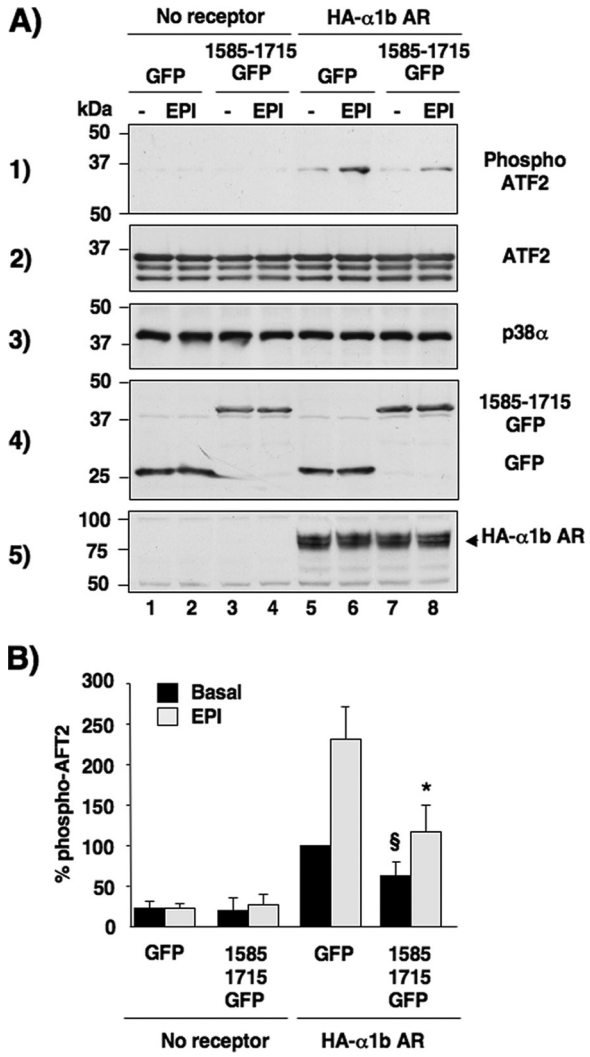

FIGURE 7. **Disruption of the AKAP-Lbc complex inhibits α_{1b}-AR-induced p38α activation.** *A*, HEK-293 cells were transfected with vectors encoding GFP or GFP-tagged AKAP-Lbc fragment 1585–1715 in the absence or presence of the plasmid encoding the HA-tagged α_1-AR. After a 24-h serum starvation, cells were incubated for 15 min with or without 10^{-4} M epinephrine (*EPI*), lysed, and subjected to immunoprecipitation using anti-p38α monoclonal antibodies. Kinase reactions were performed by incubating p38α immunoprecipitates with 1 μg of purified GST-ATF2 and in the presence of ATP. Detection of phospho-ATF2 (*panel 1*), ATF2 (*panel 2*), p38α (*panel 3*), and HA-tagged α_1-AR (*panel 5*) were performed as indicated in Fig. 1*A*. Expression of GFP as well as the GFP-tagged AKAP-Lbc fragment 1585–1715 (*panel 4*) was detected using polyclonal anti-GFP antibodies. *B*, quantitative analysis of phosphorylated ATF2 was obtained by densitometry. The amount of phospho-ATF2 was normalized to the total amount of ATF2. Results are expressed as mean ± S.E. of 3 different experiments.). §, $p < 0.05$ as compared with phospho-ATF2 levels measured in untreated cells expressing HA-α_{1b}-ARs and GFP. *, $p < 0.05$ as compared with phospho-ATF2 levels measured in epinephrine-treated cells expressing HA-α_{1b}-ARs and GFP.

we could show that the S1565A mutant of AKAP-Lbc, which is unable to bind 14-3-3, display a 2-fold higher ability to associate with endogenous PKNα when compared with wild type AKAP-Lbc (Fig. 8, *C*, *panel 1*, *lanes 2* and *3*, and *D*). This indicates that recruitment of 14-3-3 inhibits PKNα binding to AKAP-Lbc.

To determine whether this reduction of PKNα binding induced by 14-3-3 could affect the ability of AKAP-Lbc to induce p38α activation, we compared the p38 activating potential of the wild type and 14-3-3 binding deficient forms of AKAP-Lbc. We could show that deletion of the 14-3-3 binding site increases by 2.7-fold the activation of p38α induced by the anchoring protein (Fig. 8, *E*, *upper panel*, *lanes 2* and *3*, and *F*). Collectively, these results suggest that 14-3-3 exerts an inhibitory effect on the ability of AKAP-Lbc to recruit and activate the p38α signaling complex.

DISCUSSION

MAP kinase pathways are crucial mediators of several pathophysiological responses induced by α_1-adrenergic receptors (1, 9–11). Although evidence collected over the last years indicates

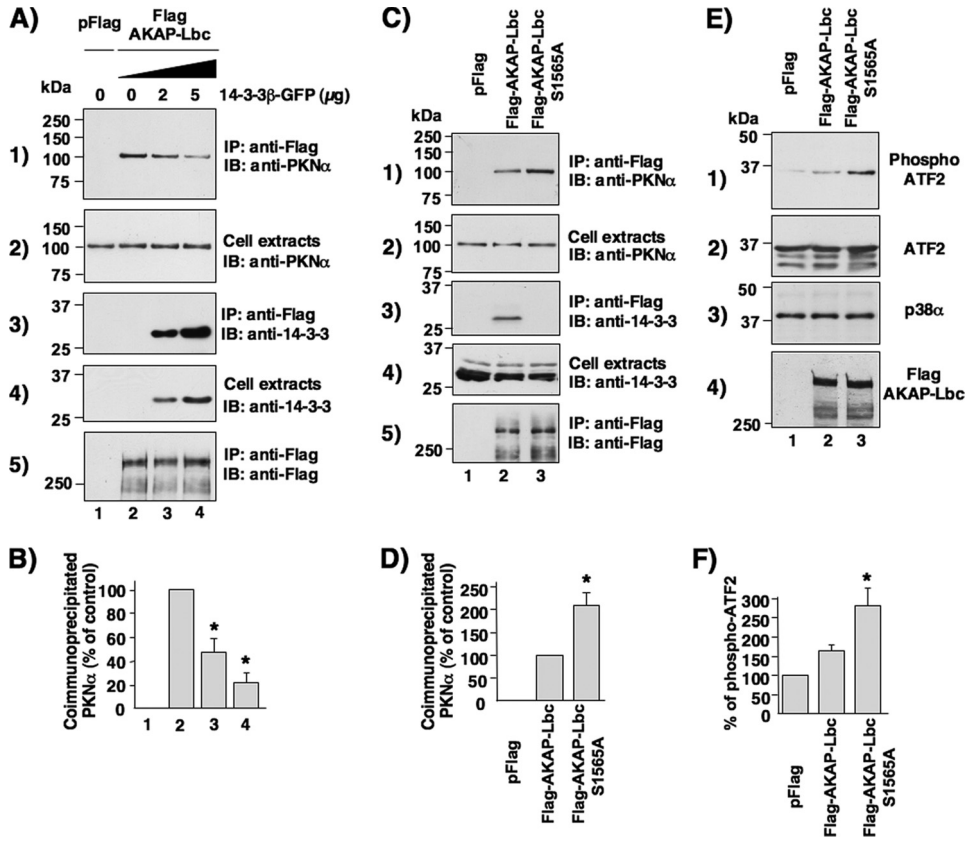

FIGURE 8. **14-3-3 inhibits the interaction between AKAP-Lbc and PKNα.** *A*, HEK-293 cells were transfected with the empty pFLAG vector or the vector encoding FLAG-AKAP-Lbc in combination with increasing amounts (indicated *above* each lane) of the plasmid encoding 14-3-3β-GFP. Cell extracts were subjected to immunoprecipitation with anti-FLAG antibodies. Western blots of the immunoprecipitates and cell extracts were revealed using anti-PKNα polyclonal antibodies (*panels 1* and *2*), anti-14-3-3β polyclonal antibodies (*panels 3* and *4*), or anti-FLAG monoclonal antibodies to detect the FLAG-AKAP-Lbc (*panel 5*). *B*, densitometry of the bands corresponding to PKNα coimmunoprecipitated with AKAP-Lbc. The amount of PKNα in the immunoprecipitates was normalized to the PKNα content of the cell extracts. Results are expressed as mean ± S.E. of three independent experiments. *, $p < 0.05$ as compared with the levels of co-immunoprecipitated PKNα measured in cells expressing only FLAG-AKAP-Lbc. *C*, extracts from HEK-293 cells transfected with plasmids encoding the empty FLAG vector, FLAG-AKAP-Lbc, or FLAG-AKAP-Lbc S1565A. Cell extracts were subjected to immunoprecipitation (*IP*) with anti-FLAG antibodies. Western blots of the immunoprecipitates and the cell extracts were revealed as indicated in *A*. *D*, densitometry of the bands corresponding to PKNα coimmunoprecipitated with AKAP-Lbc was performed as indicated in *B*. Results are expressed as mean ± S.E. of three independent experiments. *, $p < 0.05$ as compared with the levels of co-immunoprecipitated PKNα measured in cells expressing FLAG-AKAP-Lbc. *E*, extracts from HEK-293 cells transfected with the vectors encoding FLAG-AKAP-Lbc-GFP or FLAG-AKAP-Lbc S1565A. After a 24-h serum starvation, cells were lysed and subjected to immunoprecipitation using monoclonal anti-p38α antibodies. Kinase reactions and detection of phospho-ATF2 (*panel 1*), ATF2 (*panel 2*), and p38α (*panel 3*) were performed as indicated in Fig. 1*A*. Expression of the AKAP-Lbc constructs was detected using anti-FLAG monoclonal antibodies (*panel 4*). *F*, quantitative analysis of phospho-ATF2 was obtained as indicated in Fig. 1*B*. Results are expressed as mean ± S.E. of three different experiments. *, $p < 0.05$ as compared with phospho-ATF2 levels measured in cells expressing FLAG-AKAP-Lbc.

that MAPK cascades are organized in transduction modules (13), it is currently unknown how such signaling complexes are assembled and activated in response to α_1-adrenergic receptor stimulation to generate specific cellular responses. Our findings now indicate that the RhoA-selective exchange factor AKAP-Lbc recruits a signaling module containing the RhoA effector PKNα and the MLTK, MKK3, and p38α kinases, which transduce activating signals from α_{1b}-ARs down to p38α. In particular, whereas we have previously shown that α_{1b}-ARs mediate AKAP-Lbc activation through the α subunit of the heterotrimeric G protein G_{12} (2), our current model proposes that activated AKAP-Lbc promotes, through RhoA, the sequential activation PKNα, MLTK, MKK3, and p38α within the complex. On the other hand, inactivation of AKAP-Lbc-mediated p38 signaling occurs following the recruitment of 14-3-3 to AKAP-Lbc. This promotes the dissociation of the p38α activation module from AKAP-Lbc (Fig. 9) and deactivates AKAP-Lbc Rho-GEF activity (19). Therefore, AKAP-Lbc represents a molecular platform where signals that activate or deactivate p38 signaling converge.

p38 kinases were originally described to mediate cellular responses to various types of stresses (23, 26). During the last decade, however, it has become increasingly clear that members of this kinase family can participate in signaling pathways activated by of a variety of other membrane receptors, including cytokine and G protein-coupled receptors, to promote cellular functions such as proliferation, growth, inflammation, and contraction (23, 26). In particular, it was shown that activation of p38α by α_1-ARs can regulate arterial smooth muscle cell contractility (27) and promote cardiomyocyte sarcomere remodeling during cardiac hypertrophy (10).

Because of the implication of p38 kinases in such a variety of crucial responses, the molecular mechanisms involved in their activation has been the subject of intensive investigation. In this context, it is well established that GTPases of the Rho family, including RhoA, Rac1, and cdc42, are key mediators of p38

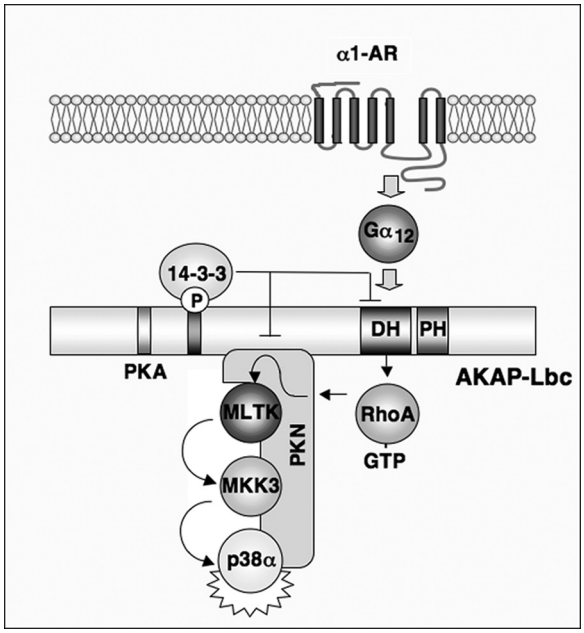

FIGURE 9. **Model for the AKAP-Lbc·p38α activation complex.** AKAP-Lbc assembles a signaling complex that includes the scaffolding protein PKNα as well as MLTK, MKK3, and p38α. The AKAP-Lbc signaling complex is activated in response to α_1-AR stimulation through a $G\alpha_{12}$-mediated signaling pathway (17). Activated AKAP-Lbc promotes the formation of RhoA-GTP, which, in turn, induces the activation of a signaling cascade that includes PKNα, MLTK, MKK3, and p38α. The recruitment of 14-3-3 inhibits AKAP-Lbc Rho-GEF activity (19), impairs the interaction between PKNα and AKAP-Lbc, and reduces p38 activation.

activation induced by membrane receptors (10, 14, 24, 28). However, how activation of Rho GTPases by upstream stimuli is translated into the activation of a specific p38 pathway is far from being entirely elucidated.

Two studies provided initial evidence that the GTPase Rac-1 and its upstream activator Tiam can recruit signaling complexes containing p38 and its upstream activating kinases. In a first study, Rac-1 was shown to directly bind to Osm, a scaffold protein that recruits MEKK3 and MKK3 (29). This complex has been implicated in hyperosmotic shock-induced p38 activation. In a second study, the Rac-1 activator Tiam was shown to recruit a p38 signaling complex formed by the scaffold protein JIP2 and the kinases MLK3 and MKK3 (30). This study, however, did not determine which extracellular stimuli activate this signaling module or whether Tiam and JIP2 can form a complex at the endogenous level.

More recently, elegant studies showed that the pro-myogenic cell surface protein Cdo can recruit two proteins named Bnip-2 and JLP, which act as scaffolds for the Cdc42 and p38, respectively (31, 32). The assembly of this signaling complex promotes Cdc42-dependent p38 activation.

In this context, our current findings that AKAP-Lbc can mediate α_1-AR-induced p38 activation through the assembly of a signaling module composed of PKNα, MLTK, MKK3, and p38α, provide new mechanistic insights on how specific signals can be vehiculated from membrane receptors to p38α. They also highlight the key function of PKNα as a scaffold protein that facilitates RhoA-dependent p38 activation. This view is supported by our results showing that PKNα can directly interact with AKAP-Lbc, MLTK, MKK3, and p38α (Fig. 5), and that silencing of endogenous PKNα in HEK-293 cells can reduce the interaction of endogenous p38α and MKK3 with AKAP-Lbc as well as AKAP-Lbc-mediated MLTK activation (Fig. 6).

Interestingly, previous studies have shown that PKNα can also promote the activation of p38γ (28) potentially through its ability to bind and activate MLTK and MKK6 (25). This suggests that PKNα can recruit a different combination of signaling enzymes to modulate the activation of different p38 isoforms. The molecular mechanisms regulating the interaction between PKNα and p38 activating kinases are currently unknown and will deserve further investigation. Based on our co-immunoprecipitation experiments, which failed to detect an interaction between p38γ and AKAP-Lbc (results not shown) one could raise the hypothesis that AKAP-Lbc might selectively stabilize the interaction between PKNα and p38α.

In addition to PKNα, three other scaffold proteins including JIP2, JIP4, and Osm have been shown to promote p38 activation (13, 29, 33). Interestingly, recent studies identified an interaction between recombinant JIP4 and a splice variant of AKAP-Lbc, called Brx, that contains only the last 1429 residues the anchoring protein (34). However, whereas these studies mapped the interaction determinants for JIP4 to the last 400 amino acids of Brx, they did not determine whether the two proteins can interact at the endogenous level.

In control experiments, we could not detect the expression of JIP4 in HEK-293 cell lysates (results not shown), suggesting that JIP4 is unlikely to be involved in the recruitment of p38α and its upstream kinases to AKAP-Lbc in this cell line. In line with this conclusion, our results indicate that deletion of the PKNα binding site (residues 1585–1715) from AKAP-Lbc abolishes the ability of the anchoring protein to recruit the p38α and its upstream kinases MKK3 and MLTK (Fig. 4). This suggests that the interaction between AKAP-Lbc and p38α is mediated by the PKNα binding domain and does not involve additional interaction sites. It is possible, however, that the ability of AKAP-Lbc and Brx to recruit PKNα or JIP4 might be influenced by the relative abundance of these scaffolding proteins in different cell types.

Our current findings support the view that AKAP-Lbc specifically mediates α_1-AR-induced p38α activation without affecting activation of ERK1/2 and JNK (Figs. 1 and 7, and supplemental Figs. S1 and S6). At the molecular level, this can be explained by the fact that AKAP-Lbc forms a complex with p38α and not with ERK and JNK (Figs. 2 and 3). Therefore, the ability of AKAP-Lbc to transmit activating signals to specific MAPK pathways is dictated by its ability to recruit specific combinations of kinases. These results suggest therefore that different signaling complexes might mediate α_1-AR-induced activation of ERK1/2 and JNK. In this respect, recent previous findings indicate that the Rho-GEF p115 can recruit a scaffolding protein named CNK1, which binds the kinases MLK2 and MKK7 to coordinate the activation of JNK1 (35). Further investigations will be required to determine whether p115 or a different signaling complex is involved in the organization of the JNK pathway downstream of α_{1b}-ARs.

Members of the 14-3-3 family regulate a variety of transduction pathways either by affecting the catalytic activity, the subcellular localization, or by regulating protein-protein interac-

tion properties of signaling molecules (36). It is well established that 14-3-3 has an antagonistic effect on the p38 signaling pathway (37, 38). This could be in part mediated by the inhibitory action of 14-3-3 on Ask1, a MAPKKK that acts upstream of p38 (39). However, our current findings now suggest that 14-3-3 can inhibit AKAP-Lbc-mediated p38α activation by inducing the dissociation of the PKNα·MLTK·MKK3·p38α complex from AKAP-Lbc (Fig. 8) as well as by inhibiting the Rho-GEF activity of the anchoring protein (19). Therefore, our results provide a new molecular explanation on how 14-3-3 proteins can impair p38 signaling inside cells.

Given the proximity of the binding sites for 14-3-3 and PKNα it is tempting to speculate that 14-3-3 recruitment might inhibit the interaction between AKAP-Lbc and PKNα by directly masking the PKNα interaction site on the anchoring protein. This is reminiscent of the mechanism through which 14-3-3 has been shown to inhibit the interaction between the pro-apoptotic protein Bad and pro-survival Bcl-2 family members (40).

In conclusion, the implications of our findings are 2-fold. First, they identify key molecular mechanisms controlling signaling specificity downstream of α_{1b}-ARs. By assembling a macromolecular signaling complex containing RhoA, PKNα, MLTK, MKK3, and p38α, AKAP-Lbc controls the specific transduction of signals from α_{1b}-ARs to p38α. Second, they provide a novel hypothesis explaining the inhibitory action of 14-3-3 on the p38 pathway, suggesting that AKAP-Lbc might represent a molecular platform integrating 14-3-3 and p38 signaling.

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