The Pennsylvania State University
The Graduate School
Intercollege Graduate Program in Genetics

REQUIREMENT FOR KM23-1 PHOSPHORYLATION IN DYNEIN
REGULATION OF TRANSFORMING GROWTH FACTOR-BETA SIGNALING

A Thesis in
Genetics
by
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km23-1 is a mammalian member of the km23/LC7/roadblock (DYNLRB) family of dynein light chains. It has been shown to bind the dynein intermediate chain in response to Transforming Growth Factor-beta (TGFβ) receptor activation, and function as a motor receptor for the intracellular transport of TGFβ signaling components. Previous work has demonstrated that the dynein-dependent intracellular events are required for Smad2 nuclear translocation and downstream TGFβ signaling transduction. Here we provide evidence that inducible phosphorylation of km23-1 is an important mechanism for dynein regulation by TGFβ signaling. In response to cellular stimuli, km23-1 undergoes multiple phosphorylations, which induce dynein complex formation and activation of downstream signaling events. Interestingly, besides TGFβ receptor phosphorylation at an early stage, km23-1 is phosphorylated by protein kinase A (PKA) at 30 minutes after TGFβ treatment, which may be a negative regulatory mechanism for the dynein complex. Our results indicate for the first time that km23-1 serves as a novel target of PKA, and that the site-specific phosphorylation of km23-1 regulates its signaling function.
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LIST OF ABBREVIATIONS

Ab antibody
AKAP A-kinase anchoring proteins
ARE activin-responsive element
BMP bone morphogenetic proteins
β beta
bp base pair
cAMP cyclic adenosine monophosphate
DIC dynein intermediate chain
DLC dynein light chain
DNA deoxyribonucleic acid
EEA1 early endosome antigen-1
ERK extracellular signal-regulated kinase
EV empty vector
GFP green fluorescent protein
hr hour
IP immunoprecipitation
JNK the c-Jun NH2-terminal kinase
kb kilobase-pair
M molar
MAPK mitogen-activated protein kinase
ml milliliter
<table>
<thead>
<tr>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT</td>
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<tr>
<td>NC</td>
<td>negative control</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>robl</td>
<td>roadblock</td>
</tr>
<tr>
<td>RSmad</td>
<td>receptor-activated Smad</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated kinase</td>
</tr>
<tr>
<td>SBE</td>
<td>the Smad-binding element</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TβR</td>
<td>TGFβ receptor</td>
</tr>
<tr>
<td>TβRI</td>
<td>TGFβ Receptor Type I</td>
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Chapter 1

Introduction

1.1 Introduction to TGFβ signaling pathways

The transforming growth factor-beta (TGFβ) superfamily is a large family of structurally related cell regulatory proteins that includes TGFβs, bone morphogenetic proteins (BMPs), activins and related proteins. Ligands of the TGFβ superfamily and their downstream signal transduction components play key roles in development and tumorigenesis. TGFβ pathways regulate a variety of cellular functions, including cell growth, recognition, differentiation, motility, apoptosis and specification of developmental fate, both early in embryogenesis and in mature tissues. TGFβ has dual roles in tumorigenesis. It was discovered and named as a “transforming” growth factor because it is able to induce malignant behavior of normal fibroblast, but it was considered as a candidate tumor suppressor a few years later due to its growth-suppressive effects on epithelial and lymphoid cells, which form the basis of the majority of human cancers. However, TGFβ production by tumor cells contributes to cancer progression, especially when the cells have lost the negative growth control imparted by TGFβ.
1.1.1 TGFβ signaling cascade

TGFβ signaling involves activation of a number of signaling pathways, most of which are regulated by phosphorylation events. There are two major intracellular signaling cascades, the Smad-dependent and the Ras/MAPK dependent pathways (Figure 1-1) 3, 9, 10.

In Smad-dependent pathway, the ligand binds to and brings together two pairs of transmembrane receptors, known as the type I (TβRI) and type II receptors (TβRII). They are structurally similar serine/threonine kinases. Ligand activation allows constitutively-active TβRII to transphosphorylate TβRI in its GS-domain. The activated TβRI then phosphorylates conserved serine residues at the C terminus of receptor-regulated Smads proteins (RSmads) such as Smad2 or Smad3 11, 12. The phosphorylated RSmads form a heteromeric complex with the common partner Smad4. Afterwards, the TGFβ-activated Smad complexes are translocated into the nucleus to participate in target gene transcription, either directly by binding to DNA consensus sites, or indirectly by interacting with other transcription factors including forkhead activin signal transducer (FAST)-1, FAST-2, c-Jun, and c-Fos 2, 3, 9, 10, 13.

TGFβ can also activate the Ras/MAPK pathways in a variety of TGFβ-sensitive epithelial cells. As our lab first demonstrated, TGFβ could activate the components of the Ras/MAPK pathways, including the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs)/stress-activated kinases (SAPKs), and p38 14-18. Additional studies demonstrated that TGFβ activation of the Ras/MAPK pathways plays critical roles in TGFβ signaling 3, 19.
Figure 1-1 TGFβ signaling cascades.

The ligand brings together two types of transmembrane receptors (TβRI and TβRII) at the cell surface. Constitutively-active TβRII transphosphorylates the GS domain of TβRI. In Smad-dependent pathways, activated kinase TβRI phosphorylates R-Smads at C-terminal serines, and these R-Smads then form a complex with Smad4. Activated heteromeric Smad complexes translocate to the nucleus, where they induce or repress transcription of target genes, mostly through cooperation with other transcription factors (TFs) and co-activators or co-repressors. TGFβ receptors also activate Smad-independent pathways, such as the Ras/MAPK pathways.
1.1.2 TGFβ receptor-interacting proteins

The intracellular TGFβ signaling responses are substantially diverse and versatile. They are regulated or modified by a series of protein-protein interaction events. Besides RSmads, a number of receptor-interacting proteins have been identified, and found to play critical roles in intracellular TGFβ signaling. For example, Smad anchor for receptor activation (SARA), which is a FYVE domain protein, binds to both TGFβ receptors and Smad2, and mediates access of Smads to the TGFβ receptor by controlling the subcellular localization of the RSmads. The FYVE finger resembles a double zinc-finger domain and binds specifically to intracellular membranes which contain phosphatidylinositol-3-phosphate. However, the mechanisms by which receptor-mediated activation promotes nuclear accumulation of Smad proteins have remained unclear. Thus, our lab was interested in the identification of additional TβR-interacting proteins, which could assist in a better understanding of TGFβ signaling regulation.

1.2 Introduction to km23-1

1.2.1 Discovery of km23-1

km23-1 was discovered in a novel screen for TGFβ receptor-interacting proteins in our lab. In this screen, the cytoplasmic domains of both TGFβ receptors were phosphorylated using an in-vitro kinase assay, and then were used as probes to screen an expressed library prepared from a highly TGFβ-responsive intestinal epithelial cell line.
Among several positive clones, km23-1 was of particular interest because it was homologous to the regulatory region of the bithorax complex (BX-C), which is involved in *Drosophila* TGFβ signaling. Further sequence alignment of km23-1 revealed that it is the mammalian homologue of the *Drosophila* protein roadblock (robl) and of the *Chlamydomonas* protein LC7, which belong to one of the three classes of cytoplasmic dynein light chains (DLC). Thus, it was hypothesized that a dynein subunit involved in TGFβ signaling might play a role in the intracellular trafficking of TGFβ signaling components. Our lab has shown that km23-1 functions both as a TGFβ signaling component and as a dynein subunit. It represents the first link between a natural growth inhibitory cytokine and the motor protein dynein.

### 1.2.2 Basic features of protein km23-1

Human km23-1 is a 96 amino acid protein encoded by a 291 base pair open reading frame. The gene is composed of 4 exons and located on human chromosome 20 (20q 11.21). The protein is expressed in the cytoplasm and has a calculated molecular mass of 11 kDa on Western blots. km23-1 is relatively conserved across different species. Human km23-1 protein shows 98% similarity to rat’s, 74% similarity to *Chlamydomonas*’, 82% similarity to *Drosophila*’s, 76% similarity to *C. elegans*’, and 93% similarity to D. Rerio’s (ZFIN gene). Another mammalian isoform of km23-1, termed km23-2, has been identified more recently, with 77% sequence identity and 93% similarity to km23-1. Both of the km23 isoforms can bind to DIC. Expression studies
showed that km23/km23-1 ubiquitously exists in human tissues, while km23-2 may be differentially expressed in a tissue-specific manner.\textsuperscript{27,28}

### 1.2.3 The requirement for km23-1 in TGFβ signaling

km23-1 interacts with the TGFβ receptor complex and undergoes rapid phosphorylation on serine residues after receptor activation. km23-1 binding to the dynein complex, as a DLC, was found dependent upon the kinase activity of TβRII.\textsuperscript{25}

km23-1 is required for mediating a number of specific TGFβ responses. Overexpression of km23-1 in TGFβ-responsive Mv1Lu cells induced specific TGFβ responses, such as Jun N-terminal kinase (JNK) activation, e-Jun phosphorylation, and an inhibition of cell growth.\textsuperscript{25} km23-1 interacts the important effector Smad2 both in vitro and in vivo. Immunofluorescence studies have shown that km23-1 and Smad2 are co-localized before Smad2 translocates into the nucleus.\textsuperscript{31} Furthermore, blockade of km23-1 expression using a small interfering RNA (siRNA) approach resulted in a decrease in cellular responses to TGFβ, including TGFβ/Smad2-dependent ARE-Lux transcriptional activity, induction of fibronectin expression, Smad2 nuclear translocation, and inhibition of cell cycle progression.\textsuperscript{29-31} These results suggested that TGFβ pathway components such as Smad2 may use km23-1 as a dynein light chain receptor for the intracellular movement from the plasma membrane towards the nucleus along microtubules.
1.2.4 km23-1 mutations in human cancer

Such an important role for km23-1 in TGFβ signaling may explain the high alteration frequency (42%) of km23-1 in human epithelial ovarian cancers, which are frequently resistant to TGFβ-mediated growth inhibition. The mutations were detected in human ovarian cancer patients using laser-capture microdissection (LCM) and nested reverse-transcription-PCR, as described by Ding et al.32. No km23-1 alterations were found in normal ovarian tissues. Further functional studies of the km23-1 mutants that were detected in cancer patients indicated that the mutants displayed a defect in dynein motor complex formation in vivo, and an inhibition of TGFβ-dependent transcriptional activity of the p3TP-lux and activin-responsive element (ARE) reporters. The results suggested that km23-1 plays an important role in TGFβ signaling. Further, mutation in km23-1 might contribute to both TGFβ resistance and human ovarian cancer32.

1.3 Introduction to cytoplasmic dynein and dynein light chains

1.3.1 Dynein overview

The dyneins represent one of the three superfamilies of known molecular motors, which drive the majority of active transport in the cell. The other two superfamilies are kinesins and myosins. Dyneins can be further classified into two forms: axonemal and cytoplasmic dynein. Axonemal dyneins are important in ciliary and flagellar movement. Cytoplasmic dyneins, which I will focus on in this section, are widely expressed in various cells and produce force for intracellular retrograde transport33-35. They play
important roles in transport of a wide variety of membranous organelles (such as endosomes, lysosomes, and other intracellular cargoes) towards the minus-ends of microtubules (MTs), the assembly and orientation of the mitotic spindle, neuronal transport, maintenance of Golgi, nuclear migration, and cell migration.

Cytoplasmic dynein is a massive, multimeric complex (Figure 1-2). All forms of dynein consist of two major domains: a motor domain and a cargo-binding domain. Both domains are comprised of different subunits that are crucial for the proper function of dynein. The motor domain contains two dynein heavy chains (HC) of ~530 kDa, which are responsible for force production and MT attachment through six AAA ATPase and MT binding sites, respectively. The cargo-binding domain of dynein is associated with the N-terminal portion of the HC. It is comprised of a variety of accessory subunits, including two dynein intermediate chains (DICs) of ~74kDa, two dynein light intermediate chains (DLICs) of ~50-60 kDa, and several dynein light chains (DLCs) of ~8-22kDa. In addition, dynactin, a multi-subunit protein complex, binds DIC directly. It is required for the attachment of dynein to some membranes in eukaryotes, and has been implicated in dynein targeting and cargo interactions.
The cytoplasmic dynein complex has two identical heavy chains (HC), which have ATP-binding and microtubule (MT)-binding sites. Through hydrolyzing ATP, HCs provide energy for dynein movement. Besides the HCs, the dynein complex has a variety of accessory subunits, including two dynein light intermediate chains (LIC) of ~50-60 kDa, two dynein intermediate chains (IC) of ~74kDa, and several dynein light chains (LC) of ~8-22kDa. There are 3 families of LC: km23, LC8, and Tctex-1.

Figure 1-2 Multimeric dynein complex
1.3.2 Three classes of dynein light chains

There are three classes of cytoplasmic DLCs identified in mammals: Tctex-1/rp3 (DYNLT), LC8 (DYNLL1), and km23/LC7/roadblock (DYNLRB)\textsuperscript{25, 35, 42, 45-47, 50, 51}. These three DLCs bind to the cytoplasmic DICs at distinct regions. Tctex-1 and LC8 bind to adjacent regions at the C-terminus of the second alternative splice region in the DIC gene. km23/LC7 isoforms directly bind to a region of DIC immediately N-terminal to the tryptophan-aspartate-repeat motifs, which is distinct from the Tctex-1 and LC8 binding sites\textsuperscript{26}. These results indicate that the three different LCs could bind to distinct regions of the same DIC.

All three DLCs have been implicated in a very wide range of interactions with other proteins, including transmembrane receptors, transcription factors, ion channels, and viruses\textsuperscript{25, 35, 42, 45-47, 50, 51}. Interestingly, the sequences in DIC, to which DLCs bind, have been found in several DLCs interacting partners\textsuperscript{35}. This observation contributed to the hypothesis that DLCs can bind the dynein complex through DICs and cargos proteins at the same time, serving as “motor receptors” for retrograde transport\textsuperscript{52-55}.

1.3.3 DLC-mediated dynein-cargo interactions

The DLCs have been increasingly shown to play crucial roles in dynein-cargo binding and to be involved in a variety of signaling pathways. Tctex-1, for example, binds to the cytoplasmic tails of rhodopsin. This interaction was abolished by mutations found in retinal degeneration\textsuperscript{56}. Tctex-1 was also found to interact with Trk neurotrophin receptors\textsuperscript{57}, bone morphogenetic protein (BMP) receptor type II\textsuperscript{58}, polio
virus receptor CD 155. Numerous interacting partners for LC8 have also been reported, such as neuronal nitric oxide synthase (nNOS), transcription factor Swallow, Bcl-2 interacting mediator (BIM), and p21-activated kinase 1 (Pak1).

There is now accumulating evidence that km23-1, which is also termed mLc7, roadblock1, and DYNLRB, can mediate dynein-cargo interactions as well. For example, it was found to be involved in the transport of mitochondrial components and the HbX protein from Hepatitis B virus. In 2003, km23-1 was identified as a novel TGFβ interacting protein by our lab. It has been shown to bind to DIC in response to TGFβ receptor activation, and over-expression of km23-1 can induce specific TGFβ responses. Furthermore, our recent data have demonstrated that km23-1 can interact with and is co-localized with endogenous Smad2, a key TGFβ signaling intermediate. km23-1 is also required for Smad2 compartmentalization and transcriptional activation. These results demonstrate that km23-1 is involved in the intracellular transport of TGFβ signaling components as a motor receptor. In this thesis, I am interested in investigating how this trafficking, mediated by km23-1 and dependent upon TGFβ signaling activation, is regulated.

1.4 km23-1 protein structure and PKA consensus sites

1.4.1 Protein structure of km23-1

km23/DLC7/roadblock (DYNLRB) was initially identified from two different sources: a molecular cloning of LC7 in the Chlamydomonas flagellar outer dynein arm,
and a mutant screen in Drosophila for larvae exhibiting sluggish motility. LC7 and Roadblock were found to share 56.7% identity and define a distinct group of dynein components. LC7 has an essential role in living cells, as mutation in its Drosophila homologue (roadblock) in larvae caused defects in both axonal transport and mitosis. The mutant flies suffered from sluggish motility, and showed severe axonal loss and nerve degenerations as a result of cargo accumulation. In 2002, our lab identified km23-1 as a novel TGFβ receptor-interacting protein and the mammalian homologue of the Drosophila roadblock and Chlamydomonas LC7.

The km23/DLC7/roadblock (DYNLRB) class of DLCs appears to be structurally different from the other two DLC classes. In the year 2000, km23-1 was found to belong to an ancient superfamily known as the MglB/roadblock superfamily, which exists in all three kingdoms of life. Members of this superfamily have been shown to be involved in NTPase regulation in bacterial and archea, and in regulating the mitogen-activated protein kinase (MAPK) signaling pathways. Recently, the three-dimensional solution structure of km23-1 was reported. Our lab has shown that km23-1 adopts the structure of a homodimer similar to that of the p14/MP1 heterodimer complex. In contrast, km23’s structure is distinct from the LC8 and Tctex-1 DLC classes, both of which also adopt homodimeric structures and have structurally related folds.
km23-1 secondary structure includes 2 α helices and 5 β-strands: α1 (2–10), β1 (17–24), β2 (29–32), α2 (36–60), β3 (66–73), β4 (76–82), β5 (86–92). Monomers are colored red and blue (monomer 1, dark blue β-strands and red/yellow α-helices; monomer 2, light blue β-strands and magenta/yellow α-helices). Three conserved serine residues are mapped to the surface of the molecule: S13 and S73 are located on a central face that includes the two-N-terminal α-helices and S32 is lying on the adjacent edge of the homodimer. (From Ilangoan U, Ding W, Zhong Y et al. J Mol Biol. 2005 Sep 16;352(2):338-54)
1.4.2 Conserved serine residues and PKA consensus sites

km23-1 has been shown to be phosphorylated on serine residues after TGFβ receptor activation and bind to the DIC in response to this phosphorylation. There are a total of six serine residues in the km23-1 sequence. Three of them are conserved across species, including serine residue13 (S13), serine residue 32 (S32) and serine residue 73 (S73), suggesting that they are structurally important to km23-1 function. The most interesting feature of this three-dimensional structure is that the conserved residues, including the three serine residues, are mapped to a central face of the molecule and to the adjacent edge that includes the two-N-terminal α-helices. S13 and S73 are located on the surface that includes the two-N-terminal α-helices, and S32 is lying on the adjacent edge. This observation suggests that the central face of km23-1 may be involved in DIC binding, and that the phosphorylation of the three conserved serines, or at least some of them, by TGFβ may regulate this event.

We found that PKA is a potential kinase that can phosphorylate km23-1 by using NetPhos 2.0, NetPhosK 1.0, and Prosite Motif Search. Previous studies have shown that PKA plays important roles in both TGFβ signaling and dynein function regulation. Thus, we were interested in testing the hypothesis that PKA could phosphorylate km23-1 in response to TGFβ receptor activation and that this phosphorylation event could regulate km23-1 functions in intracellular transport.
1.5 Interaction between PKA and TGFβ signaling

It has been reported that TGFβ can activate several other signaling pathways, such as phosphatidylinositol 3-kinase (PI-3K), protein kinase A (PKA) and protein kinase C (PKC), among others. We were most interested in the PKA pathway because it regulates TGFβ signaling reciprocally.

1.5.1 Introduction to the PKA pathway

PKA was originally used to identify a family of enzymes whose activity was dependent on the intracellular level of cyclic AMP (cAMP). Each PKA is a cytosolic, tetrameric holoenzyme that consists of two regulatory subunits associated with two catalytic subunits. In the conventional cAMP-dependent pathway, the holoenzyme remains intact and catalytically inactive under low levels of cAMP. When the intracellular concentrations of cAMP rise following activation of adenylate cyclases by extracellular ligands, cAMP will bind to the regulatory subunits of PKA and induce a conformational change. Then the holoenzyme will dissociate into a regulatory subunit dimer with 4 cAMPs bound and 2 catalytically free subunits. The catalytic subunits then become catalytically active and are ready to phosphorylate serine and threonine residues on specific substrates. However, more recently, Zhong et al. demonstrated that PKA could also be activated without cAMP elevation. This group found that the α-catalytic subunit of PKA is constitutively associated with IκB, and that degradation of IκB could stimulate PKA-catalytic subunit kinase activity.
1.5.2 A-kinase anchoring proteins (AKAPs)

Subcellular localization of PKA is mainly mediated by anchoring of the regulatory subunits through a family of adapter proteins called A-kinase anchoring proteins (AKAPs). A number of studies have demonstrated that PKA is compartmentalized in different subcellular locations through the interaction between PKA regulatory subunits and adaptor protein AKAPs. Each AKAP has at least two classes of functional domains: a conserved anchoring domain which binds the regulatory subunit dimer of the PKA holoenzyme and a unique targeting domain which directs the AKAP-PKA complex to distinct subcellular locations through interactions with other proteins, membranes, or cellular organelles.

1.5.3 Interaction between PKA and TGFβ signaling

The PKA pathway induces many effects on cells similar to TGFβ such as regulation of cell cycle, proliferation, chromatin condensation, and decondensation, and in particular, stimulation of fibronectin production by activation of gene transcription. It is also involved in the regulation of microtubule dynamics, exocytotic events in epithelial cells, β-aderenergic signaling in the cardiovascular system and in adipose tissue, and regulation of steroidogenesis and reproductive function, as well as in modulation of immune response and of other effects of hormones, neurotransmitters, and various paracrine ligands.
There are several lines of evidence demonstrating the interactions between TGFβ signaling pathways and PKA. Sharma and colleagues have demonstrated that TGFβ inhibits type I inositol 1,4,5-triphosphate receptor expression and enhances its phosphorylation in Mesangial cells mediated by PKA. It was also found that TGFβ could stimulate PKA in mesangial cell. In this paper, they used a specific PKA kinase assay and found that PKA activity was increased by 3-fold within 15 minutes of TGFβ-1 treatment of the mesangial cells, and by 2-fold in TGFβ-sensitive mink lung epithelial cells. This enhanced kinase activity was completely reversed by the inhibitory peptide for PKA (1uM). TGFβ-1 treatment has also been found to stimulate translocation of the α-catalytic subunit of PKA to the nucleus, starting 15 minutes after the treatment. Further, inhibition of PKA by H-89, a relatively specific inhibitor of PKA, has been found to completely inhibit TGFβ-induced cAMP response element-binding protein (CREB) phosphorylation and to attenuate TGFβ-1 stimulation of the fibronectin promoter. Both of these events require a consensus CRE (TGACGTA) site. Besides TGFβ-1, PKA has also been found to be stimulated by other TGFβ superfamily members, such as activin and bone morphogenetic protein-2.

Zhang et al. in 2004 demonstrated a novel interaction between Smad-dependent TGFβ and PKA signaling pathways. This group showed that PKA activity increased more than two-fold within 15 min of TGFβ treatment and remained elevated for 60 min. This PKA activation was independent of changes in intracellular cAMP or IκB levels. However, their results suggested that a complex was formed endogenously, comprised of Smad proteins and the regulatory subunit of PKA, with release and activation of the...
catalytic subunit from the holoenzyme. The interaction between Smad and PKA was found to be facilitated by AKAPs demonstrated by the use of the AKAP inhibitor peptide Ht31. Furthermore, this group showed that PKA activation was required for TGFβ activation of CREB, induction of p21^{Cip1}, and inhibition of cell growth.\(^7^2\)

Bioinformatics research provided very interesting results suggesting a novel mechanism of involvement of PKA in TGFβ signaling. This paper investigated the domain architecture of an AKAP from *Caenorhabditis elegans* (AKAP_{CE}) using sequence-based bioinformatics methods.\(^9^0\) The results showed that AKAP_{CE} shares two domains with the Smad anchor for receptor activation (SARA): a TGFβ receptor binding domain and a FYVE-finger. The C-terminal TGFβ receptor binding domain has several well-conserved sequence motifs, indicating that this 530 amino acid domain is functionally important. The paper proposed that AKAP_{CE} may interact with TGFβ signaling pathways by recruiting PKA to a receptor belonging to the TGFβ receptor superfamily.\(^9^0\)

Further, inhibition of PKA by H-89, a relatively specific inhibitor of PKA, has been found to completely inhibit TGFβ-induced cAMP response element-binding protein (CREB) phosphorylation and to attenuate TGFβ-1 stimulation of the fibronectin promoter which requires a consensus CRE (TGACGTA) site.\(^7^1\)
1.6 Dynein regulation by PKA

The PKA signaling cascade has been shown to play an important role in the regulation of dynein function in intracellular transport\textsuperscript{74, 75, 91, 92}. However, the exact mechanisms of this regulation are poorly understood. In pigment cells, transport of pigment granules in melanophores throughout the cytoplasm is mediated by molecular motors, including cytoplasmic dynein\textsuperscript{93, 94}. It has been recently found that this intracellular transport is tightly regulated by PKA signaling\textsuperscript{74}. Kashina \textit{et al.} has demonstrated that PKA and the scaffolding protein AKAP associates with pigment granules in cells\textsuperscript{74}. Furthermore, they found that the PKA regulatory subunit forms complexes with cytoplasmic dynein, leading to pigment aggregation, and that the removal of PKA from granules causes dissociation of dynein and disruption of dynein-dependent pigment aggregation\textsuperscript{74}. The mechanisms underlying the PKA signaling cascade regulating pigment dispersion or aggregation are likely to involve phosphorylation and dephosphorylation\textsuperscript{74, 94}. This paper concluded that such cytoplasmic protein complexes, which include motor proteins such as cytoplasmic dynein and signaling molecules such as PKA subunits, maybe involved in different components of intracellular transport. This protein complex arrangement appears to be the most efficient way to switch between different kinds of transport in response to specific signals\textsuperscript{74}.

PKA also phosphorylates p150\textsuperscript{Glued}, a subunit of the dynactin complex, and regulates the binding of p150\textsuperscript{Glued} to MTs\textsuperscript{92}. Dynactin is believed to be involved in cargo
binding. p150\textsuperscript{Glued} has also been identified as a specific binding partner for the DICs of cytoplasmic dynein and is able to dynamically interact with MTs\textsuperscript{92}. Interestingly, it has been found that drug forskolin that activates PKA, reduced microtubule binding by PKA, while the PKA inhibitor H89 enhanced this binding. The relevant phosphorylation site on p150\textsuperscript{Glued} was identified as Serine19, using phospho-amino acid analysis. S19 is conserved and is the consensus site for PKA. Its phosphorylation mutants S19A and S19E have been shown to recapitulate the corresponding phenotypes in vitro and in vivo\textsuperscript{92}.

Thus, we were interested in testing whether PKA can phosphorylate km23-1, and further, whether this phosphorylation could regulates km23-1 activity in TGF\textbeta signaling and/or in cytoplasmic dynein function.

Collectively, TGF\textbeta signaling transduction requires highly regulated intracellular trafficking and compartmentalization of the signaling complexes. In contrast, perturbation of dynein-mediated intracellular trafficking of TGF\textbeta signaling complexes might disrupt downstream transcriptional events, and could result in various disease states, including cancer. Our lab has identified that km23-1, a cytoplasmic dynein subunit, undergoes rapid phosphorylation after T\textbeta R activation\textsuperscript{25}. Recent results have shown that km23-1 is required for the recruitment of Smad2-dependent TGF\textbeta signaling complex for intracellular transport along MTs\textsuperscript{29,30,31}. Thus, we were interested to test if site-specific phosphorylation(s) of km23-1 is(are) required for its function in dynein regulation of TGF\textbeta signaling. Human study has identified high frequency (42%)
alteration rate of km23-1 in TGFβ resistant ovarian cancer\textsuperscript{30,32}. Therefore, this study on km23-1 phosphorylation might help us not only to further understand TGFβ signaling mechanism, but also to develop km23-1 based anti-cancer diagnostics and therapeutics.
Chapter 2

Methods and Materials

2.1 Reagents

TGFβ1 was purchased from R&D Systems (Minneapolis, MN). The FuGENE 6 transfection reagent was from Roche Applied Science. The Dual-Luciferase Reporter Assay System (E1960) was purchased from Promega (Madison, MI). The QuickChange Site-Directed Mutagenesis Kit was from Stratagene (La Jolla, CA. Cat#200518).

The mouse IgG was from Sigma-Aldrich, and the rabbit IgG was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-DIC monoclonal Ab (MAB1618) was from Chemicon (Temecula, CA). The rabbit TβRII Ab (SC-220), and the rabbit TβRI Ab (SC-389) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-FLAG M2 (F3165) Ab was from Sigma-Aldrich (St. Louis, MO). The anti-V5 Ab (R960 25) was obtained from Invitrogen (Carlsbad, CA). [32P]orthophosphate (NEX-053), γ-[32P]ATP (BLU002H), and [3H]thymidine (NET-027X) were from PerkinElmer Life Sciences (Boston, MA). The PKA inhibitor H89 was from Calbiochem.
2.2 Constructs

The V5-tagged RI construct was generated by inserting the Alk-5 RI cDNA fragment (provided by K. Miyazono lab, University of Tokyo, Tokyo, Japan), which was prepared by NotI and XhoI restriction enzyme digestion, into pcDNA3.1/V5-His (V-810-20; Invitrogen). The HA-tagged RII construct was provided by Dr. J. Wrana (Samuel Lundenfeld Res. Institute, Toronto, Canada). The Flag-tagged S13A-km23-1, S32A-km23-1, S55A-km23-1, S73A-km23-1, and S32AS73A-km23-1 constructs were produced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA. Cat#200518) following the manufacturer’s protocols. S32A-km23-1, S55A-km23-1, S73A-km23-1, and S32AS73A-km23-1 constructs are constructed by Qian Tang (Mulder lab). A construct encoding His-tagged human km23-1 was generated by PCR amplification of km23-1 coding region and insertion into the NdeI and BamHI sites of the bacterial expression vector pET15b (Novagen, Madison, WI) as described previously. His-tagged S13A-km23-1, S32A-km23-1, S55A-km23-1, S73A-km23-1 and S32AS73A-km23-1 constructs were produced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) from His-tagged WT km23-1. All constructs were verified by DNA sequencing in both directions.

2.3 Cell culture

Mv1Lu (CCL-64) and 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco's Modified Eagle's Medium
with 10% (v/v) heat-inactivated fetal bovine serum. Cells were maintained in 5% CO\textsubscript{2} at 37 °C. Cultures were routinely screened for mycoplasma using Hoechst 33258 staining\textsuperscript{25}.

2.4 Antibody production

\textbf{km23-1 Ab}

The rabbit polyclonal km23 anti-serum was prepared against the following sequence: GIPIKSTMDNPTTTQYA (corresponding to amino acids 27–43) of human km23-1 (hkm23-1) (Strategic BioSolutions, Newark, DE, or Covance Research Products, Denver, PA) as described previously\textsuperscript{29}.

\textbf{Serine-73 phospho-specific km23-1 Ab}

The anti-serine-73 phospho-specific antibody was custom-generated by Anaspec (San Jose, CA) in rabbits against the phosphospecific serine-73 peptide (TFLRIR (pS) KKNE-NH\textsubscript{2}) conjugated to keyhole limpet hemocyanin (KLH). The anti-serine-73 phosphospecific antibody was further purified by positive-selection affinity chromatography using the phosphor-peptides and by negative-depletion using nonphospho-peptides (TFLRIR S KKNE-NH\textsubscript{2}).

2.5 Transient transfection, In vivo phosphorylation, IP, Westerns

These assays were performed essentially as described previously\textsuperscript{25}.
2.6 In vitro kinase assays

**In vitro phosphorylation of km23-1 by TGFβ receptors**

An in vitro kinase assay using pET15b-His-km23, pET15-His-RII-Flag, and pET30-His-RI-S was performed as described previously (Bassing et al., 1994; Tang et al., 2002).

**In vitro phosphorylation of km23-1 by PKA**

To measure km23-1 phosphorylation by PKA, 12ug purified His-km23-1 fusion protein and 10uCi γ-32P ATP (from PerkinElmer) were added to buffer provided by PKA assay kit (Upstate). For PKA assays, three concentrations (0.01ug, 0.02ug, or 0.04ug) of the catalytic subunit of PKA (Upstate) were added per reaction. 5uM PKI (Bachem) was included in one reaction with 0.02ug PKA as a negative control. Kinase reactions were conducted at 30°C for 30 minutes and terminated by the addition of SDS-PAGE sample buffer. The proteins were subjected to 15% SDS-PAGE and 32P-labeled fusion proteins were detected by autoradiography.

2.7 Luciferase reporter assays

TGFβ-dependent ARE-lux reporter assays were done in Mv1Lu cells. Mv1Lu cells were co-transfected with ARE-Lux along with forkhead activin signal transducer-1, (FAST-1), and 500ng/ml of either wild-type (wt) km23-1 or S73A-km23-1. All assays were done in triplicate as described previously.
Chapter 3

Results

3.1 Conserved serine residues are required for km23-1 phosphorylation after TβR activation

It has been shown that km23-1 is a TGFβ signaling component. It is associated with the activated TβR complex endogenously, and is phosphorylated on serine residues in response to TβR activation as demonstrated by phospho-amino acid analysis. This finding is consistent with the fact that TβRs are serine/threonine kinases. In addition, we have found that a kinase active TβRII is required for km23-1 phosphorylation and for the DIC binding function of km23-1. Blockade of km23-1 using an siRNA has significantly decreased specific downstream TGFβ effects, such as induction of fibronectin expression and inhibition of cell cycle progression. The emerging picture from these results is that TGFβ signaling leads to km23-1 phosphorylation(s), and that specific phosphorylation events are required for downstream TGFβ signaling, which includes dynein complex formation and intracellular transport of TGFβ components.

As mentioned earlier, there are three conserved serine residues (S13, S32, and S73), and one partially conserved serine residue (S55), in km23-1 protein sequence. We therefore generated the S→A (Serine to Alanine) phosphorylation mutants S13A, S32A, S55A, and S73A by site-directed mutagenesis method (Figure 3-1 A). Of these four
serine residues, three (S32, S55, and S73) are located in exon 3, which has been shown to be required for km23-1 activity of binding to DIC. In order to examine whether two of the conserved serine residues, S32 and S73, and one partially conserved serine residue, S55, are critical sites for phosphorylation, in vivo phosphorylation assays were performed by transient expression of TβRI and TβRII in 293T cells, together with either wild-type km23-1 (wt km23-1) or the indicated km23-1 mutants, as previously described. As shown, wt km23-1 was not phosphorylated in the absence of the TGFβ receptors, TβRI and TβRII, (Figure 3-1B, top panel, lane 2), but in their presence, it was (Figure 3-1B, top panel, lane 3). Such phosphorylation was partially reduced upon expression of S32A-km23 or S55A-km23 (Figure 3-1B, top panel, lanes 4 and 5, respectively), and significantly reduced upon expression of S73A-km23 (Figure 3-1B, top panel, lane 6). Densitometric analysis, shown in Figure 3-1B, left panel, revealed that the phosphorylation levels of S32A, S55A, and S73A-km23 were reduced by 31%, 27% and 53%, respectively, relative to that of wt km23. Such findings demonstrate that serine residues S32, S55, and S73 are critical sites for phosphorylation by TGFβ receptors.
Figure 3-1A Schematic of 4 S-A mutants of km23-1.

Wt km23-1 has three conserved serine residues (S13, S32, and S73) and one partially conserved serine (S55). All of them have high predicted scores (above 0.9 with the maximum of 1) for potential phosphorylation. Three of them (S32, S55, and S73) are included in exon3, which is required for km23-1 DLC function and TGFβ-dependent transcription activity. S→A phosphorylation mutants were created by Qian Tang (Mulder lab) using a site-directed mutagenesis kit from Stratagene.\(^{25}\)
Figure 3-1B Three serine residues in km23-1 are phosphorylated upon activation of the TGFβ receptors.

(Left) Serine mutations of km23-1 (S32A, S55A, and S73A) block in vivo phosphorylation of km23-1 by the TGFβ receptors. 293T cells were transiently transfected as indicated in the legend above the gels. Twenty hours after transfection, cells were incubated in serum-free, phosphate-free medium for 30 min, labeled for 3 h with γ-32P-ATP, and treated with TGFβ1 during the last 15 min of the labeling period. The cells were lysed and immunoprecipitates were prepared using an anti-Flag antibody. The results of the in vivo phosphorylation were analyzed by SDS-PAGE and autoradiography and are shown in the upper gel; the lower gel demonstrates equal expression of the transfected km23-1-Flag variants via Western blot. (Right) Result of the densitometric scan of the autoradiogram shown in the left panel.
3.2 km23-DIC complex formation is regulated by TGFβ signaling

As mentioned in the introduction, km23-1 is the mammalian homologue of the chl/LC7 and Drosophila robl proteins, which are DLCs and able to bind to DIC. Since km23-1 is phosphorylated after TβR activation, it was of interest to determine whether the association between km23-1 and DIC could be changed by TβR activation. It has been shown in Tang et al., 2002 that km23-1 binding to DIC requires RII kinase activity and was mildly increased after TGFβ treatment in MDCK cells. To further confirm this induction in highly TGFβ-responsive Mv1Lu cells, we performed IP/blot analyses by using anti-DIC as the IP antibody and anti-FLAG as the blotting antibody. As shown in Figure 3-2, lanes 1-3 of the top panel, TGFβ1 (10ng/ml) induced a rapid recruitment of km23-1 to endogenous DIC. A basal level of interaction between km23-1 and DIC before TGFβ treatment was detectable, but at very low as shown in lane 1. This association was increased at 5 min (top panel lane 2), and significantly elevated at 15 min (top panel lane 3) after TGFβ addition. The bottom panels demonstrate roughly equal expression and loading of DIC and km23-flag. This result shows that TGFβ rapidly induced the recruitment of km23-1 to DIC in highly TGFβ-responsive Mv1Lu cells. It suggests that km23-1’s activity as a DLC could be highly stimulated by TGFβ signals. We further questioned whether site-specific phosphorylation of km23-1 is the underlying mechanism for this regulation, as addressed in Chapter 4.
Figure 3-2 TGFβ stimulates the recruitment of km23 to DIC in Mv1Lu cells.

Mv1Lu cells were transiently transfected with wt-km23-Flag. Twenty four hours after transfection, cells were incubated in serum-free medium for 60 min before addition of TGFβ (10ng/ml) for 0, 5, or 15 min. Cell lysates were collected and IP’d using a monoclonal anti-DIC antibody, followed by immunoblot analysis with an anti-Flag antibody (top)\textsuperscript{25}. Western blot analysis with anti-DIC (middle) or anti-FLAG (bottom) showed equal protein expression and loading.
Chapter 4

Results

4.1 Serine 32 and serine 73 are required for the recruitment of km23-1 to DIC after TβR activation

Since we have previously shown that TGFβ stimulates km23-DIC complex formation, we hypothesized that km23-1 phosphorylation after TGFβ activation is required for the DIC binding function of km23-1. It was conceivable that the phosphorylation mutants (S13A, S32A, S55A, and S73A) of km23-1 might be deficient in their interaction with DIC. Accordingly, we performed IP/blot analysis in 293T cells using anti-DIC as the IP antibody, and anti-Flag as the blotting antibody after transient expression of either wt-km23 or the indicated km23 mutants together with the TGFβ receptors (Figure 4-1). While the basal interaction between the km23-1 mutants and DIC has been shown previously, here we show TGFβ–mediated interactions with the mutants. Figure 4-1 demonstrated that the S32A-km23 and S73A-km23 mutants significantly blocked the interaction of km23-1 with DIC upon TGFβ activation (Top left, lane 4 and 6), while the S13A-km23 and S55A-km23 mutants could still bind to DIC (lane 3 and 5). No specific band was detectable in the EV and IgG control lanes. Other panels in Figure 4-1 depict equal expression and loading for DIC, RII, RI and km23 proteins. These findings suggest that S32 and S73 could be critical phosphorylation sites.
for the recruitment of km23-1 to DIC after TβR activation, while S13 and S55 are not crucial for km23-DIC binding.
Figure 4-1 S32A and S73A mutants of km23-1 disrupt binding to DIC.

Total cell lysates were prepared from 293T cells that had been transfected with wt km23-1 or four S→A mutants of km23-1 together with two TβRs. Cell lysates were then IP’d with a monoclonal DIC Ab overnight. The interacting proteins were analyzed by SDS-PAGE and Western blotting using an anti-Flag antibody (Top left). Lysates from empty vector-transfected cells and normal mouse IgG were used as negative controls (lanes 1 and 7, top left). Equal expression of DIC is shown at the bottom left. Equal inputs and expression of RI-V5, RII-HA, and Flag-tagged proteins were verified by Western blotting (right). Results are representative of two experiments.
4.2 PKA activity is required for DIC binding of km23-1 after TGFβ treatment

Since our results have show that serine residues S32 and S73 are both important phosphorylation sites in km23-1, it is possible that there are two separate phosphorylation events for km23-1, downstream of TβRs activation. To identify other possible kinases that may phosphorylate km23-1 besides the TβR kinase, we used NetPhos 2.0, NetPhosK 1.0, and Prosite Motif Search and found several potential phosphorylation sites for a number of common kinases, including protein kinase A (PKA), protein kinase C (PKC), and casein kinase II (CKII) (Figure 4-2A). Among these sites, two of them are potential PKA consensus phosphorylation sites, which were identified as K/R-R-X1-S/T-X2 (more stringent) or R-X1/2-S/T-X2 (less stringent)\(^{95,96}\). One of the PKA consensus sites covers S13 and the other starts with S73. As mentioned earlier, S13 and S73 are conserved and are located on the surface, which suggested that they might play important roles in protein function. Previous study has shown that PKA plays important roles in both TGFβ signaling and dynein function regulation\(^{71-75}\). Thus, we are interested in testing the hypothesis that PKA activity is required for km23-1 function in terms of DIC binding.

To test if PKA activation is required for km23-1 functions in TGFβ signaling, we performed IP/blot assays as described in Figure 3-2, following treatment with increasing concentrations of H89, a specific inhibitor of the PKA catalytic subunit. With only the vehicle DMSO, TGFβ treatment for 15 minutes induced near by a three-fold increase in the interaction between km23-1 and DIC (Figure 4-2B top panel, lanes 1 and 2). H89 (0.2μM) (Figure 4-2B lane 3 and 4) inhibited the induction of DIC binding to km23-1, to
less than two-fold. 3uM and 10uM H89 completely blocked the km23-DIC interaction (Figure 4-2B lane 5 to 8). The middle and the bottom panels are the expression and loading controls for DIC and transfected km23-Flag proteins. The relative units shown below the gel are densitometry scan values for km23-Flag levels (first panel) normalized for total immunoprecipitated DIC (second panel). This result shows that inhibition of PKA kinase activity by H89 blocks TGFβ-induced km23-1 binding to DIC in a dose-dependent manner. It suggests that PKA is activated upon TGFβ ligand binding and this activation is required for km23-mediated dynein complex formation.
Figure 4-2A Schematic of human km23-1 sequence

Wt km23-1 consists of four exons. Exon 3 is the largest and encodes a region of 56 amino acids. Potential phosphorylation sites for PKA, PKC, and/or CKII were predicted using NetPhos 2.0, NetPhosK 1.0, and Prosite Motif Search. Four serine residues conserved among the mammalian km23-1 forms are boxed. S13 and S73 are located in two separate PKA phosphorylation consensus sites. (Modified from Ding W et al, Cancer Res 2005; Aug 1;65(15):6526-33)
Figure 4.2B The PKA inhibitor H89 blocks the TGFβ-mediated binding of km23 to DIC in a dose-dependent manner.

Mv1Lu cells were transiently transfected with km23-Flag. Twenty four hours after transfection, cells were incubated in SF medium for 60 min, and pretreated with either PKA inhibitor H89 at increasing concentrations (0.2uM, 3uM, and 10uM), or the vector DMSO for 30 min. Thereafter, half of the plates at each H89 concentration were treated with TGFβ-1 (10 ng/ml) for 15 min. Top, cell lysates were IP’d using a monoclonal anti-DIC antibody, followed by blotting analysis with an anti-Flag Ab.
Lower panels show expression and loading controls by IP/blot with anti-DIC or by Western blot analysis with anti-Flag Ab. The relative units (top panel) are the ratios of densitometry scan values for km23-Flag levels divided by total immunoprecipitated DIC.
4.3 km23-1 phosphorylation on Serine 73 is detectable using a phospho-specific antibody.

As mentioned previously, Serine 13 (S13) and Serine 73 (S73) are PKA consensus sites in the km23-1 sequence, while S73 but not S13 is required for the DIC binding function of km23. In addition, the PKA inhibitor H89 could block the association between km23-1 and DIC as the S73A phosphorylation mutant does. Therefore, we hypothesize that S73 is the PKA phosphorylation site in km23-1. To further investigate km23-1 phosphorylation by PKA, we generated S73 phospho-specific Ab (S73-P-km23) against the phospho-S73 peptide (TFLRIR (pS) KKNNE-NH2).

Using a S73 phospho-specific km23-1 Ab, we determined the kinetics for phosphorylation of both transfected (Figure 4-3A) and endogenous km23-1 in the highly TGFβ-responsive Mv1Lu cell line (Figure 4-3B). The cells were treated with TGFβ-1 (10ng/ml) for 0, 2, 5, 15, or 30 mins, and cell lysates were collected and IP’d using a Flag Ab for transfected protein or a km23-1 Ab for endogenous protein. Total IP’d protein was then blotted using the anti-S73 phospho-km23 Ab. To our surprise, the results showed that km23 is phosphorylated on S73 at 30 minutes after TGFβ treatment.

This phosphorylation occurred later than expected, because our previous results showed that PKA activity was required for the association of DIC with km23 as early as 15 min. Previously published work provided some explanations. For example, Zhang et al. demonstrated that PKA activity at 30 min after TGFβ treatment was as high as that at 15 min (doubled compared to basal levels), but started to decrease by 60 min. In addition, this report showed that the association between PKA and Smads was constant.
from 15 min to 30 min after TGFβ treatment, but this signaling complex was no longer detectable by 60 min after TGFβ. Thus, it is possible that PKA is activated by TGFβ as soon as 15 min after TGFβ treatment, and functions to stabilize the dynein-km23-Smad complex. By 30 min, it may then phosphorylate km23-1, which in turn, may result in disassociation of the motor-signaling complex.

Since our lab has shown that km23-1 is phosphorylated after TGFβ as early as 15 min, we hypothesized that there may be at least two phosphorylation events occurring. One event may occur early after TGFβ treatment and represent direct phosphorylation by the TβRs, and the other may occur later and be mediated by PKA. Therefore, we continued to investigate whether PKA could directly phosphorylate km23-1.
Figure 4-3 An S73 phospho-specific antibody detects km23-1 phosphorylation after TGFβ treatment. (A) Mv1Lu cells were transfected with km23-Flag, and treated with TGFβ (10 ng/ml) for the indicated times. Cell lysates were subjected to IP using a Flag Ab, and blotted using the S73 phospho-specific anti-km23 antibody (S73-P-km23). Lower panel, Western blot analysis showing equal expression of km23-Flag using anti-Flag. (B) Mv1Lu cells were treated with TGFβ1 (10 ng/ml) for the indicated times. Endogenous km23-1 was IP’ed using anti-km23-1 (against amino acids 1-15), and blotted using S73-P-km23. Lower panel, Western blot analysis showing equal expression of endogenous km23-1 using anti-km23-1 (against amino acids 27-43).
4.4 PKA directly phosphorylates km23-1

To confirm that PKA is a direct kinase of km23-1, we performed in vitro kinase assays with the catalytic subunit of PKA as the kinase, and purified His-km23 protein as the substrate. The top panel of Figure 4-4A shows the direct phosphorylation of km23-1 by PKA in a dose-dependent manner (top panel, lanes 1-3). PKA inhibitory peptide (5uM) completely blocked the phosphorylation of km23-1 by PKA (top panel, lane 4). Lower panels show Coomassie blue staining of the expression of His-km23 fusion proteins and equal loading of the PKA catalytic subunit. This data demonstrates that PKA is able to directly phosphorylate km23-1 in vitro.

To confirm that km23-1 is phosphorylated by PKA at S73, we repeated the above in vitro kinase reaction and blotted the membrane with the S73 phospho-specific anti-km23-1 Ab (S73-P-km23). As shown in Figure 4-4B, similar to the results in Figure 4-4A, phosphorylation detected by the S73-P-km23 Ab is also dependent upon the dose of PKA. From lanes 1-3 of the top panel, it is clear that the level of S73 phosphorylation was increased with the addition of PKA kinase at concentrations from 0.01ug, 0.02ug to 0.04ug. Lane 4 was the negative control without substrates added.

To further confirm that km23-1 phosphorylation at S73 was PKA specific, we tested whether H89 could block S73 phosphorylation at 30 min after TGFβ treatment in Mv1Lu cells (Figure 4-4C). Without H89, phosphorylation of km23-Flag was detected using the S73-P-km23 Ab at 30 min after TGFβ treatment (top panel, lane 3). No corresponding band was observed with H89 treatment (top panel, lane 5). This result is
consistent with Figure 4-3, and further confirmed that PKA directly phosphorylates km23-1 on Serine 73 in response of TGFβ treatment.
**Figure 4-4A PKA directly phosphorylates km23-1 in vitro.**

Purified His-km23-1 fusion proteins were used as substrates in an in vitro kinase reaction using the recombinant catalytic subunit of PKA in the presence of [γ-32P] ATP. The top panel shows that phosphorylation of His-km23-1 by PKA is dependent upon the kinase dose ranging from 0.01ug to 0.02ug to 0.04ug (lanes 1-3), and is completely abolished by inclusion of PKI (lane 4). Equal levels of His-km23-1 fusion proteins and of the catalytic subunit of PKA were revealed by Coomassie blue staining in the lower panels.
Figure 4-4B The S73 phospho-specific Ab detects in-vitro PKA phosphorylation of km23-1.

Purified His-km23-1 fusion proteins were used as substrates in an in vitro kinase reaction using the recombinant catalytic subunit of PKA. After pull down of Ni-NTA agarose followed by SDS-PAGE, the His-fusion proteins were blotted using the S73 phospho-specific km23-1 Ab. The top panel shows that S73 phosphorylation of His-km23 by PKA is increased with elevated amounts of kinase. Lane 4 is the negative control without His-km23-1 substrate. Equal levels of His-km23 fusion proteins (lanes 1-3) are shown using the anti-km23 Ab prepared against amino acids 27-43.
Mv1Lu

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Figure 4-4C PKA inhibitor H89 blocks km23-1 phosphorylation on Serine 73.

Mv1Lu cells were transfected with km23-Flag, and treated with TGFβ-1 (10ng/ml) for the indicated times. Cells in Lanes 4 and 5 were treated with H89 for 30 minutes before TGFβ treatment. Cell lysates were subjected to IP using a Flag Ab, and blotted using an S73-P-km23 Ab. Lane 6 is the IgG control. Lower panel shows equal expression of km23-Flag using an anti-Flag Ab.
4.5 TGFβ signaling stimulates an endogenous interaction between PKA and km23-1.

Our results have indicated that PKA is a direct kinase of km23-1. To determine whether there is a protein-protein interaction between km23-1 and PKA, we performed IP and Western blotting with Mv1Lu cell lysates. Cells untreated or treated with TGFβ at different time points were harvested, IP'd with Ab to the RIβ regulatory subunit of PKA, and blotted with an anti-km23-1 Ab (against 27-43 amino acids). Four regulatory subunit isoforms of PKA have been identified and have been shown to possess different tissue distributions. We used the RIβ isoform because its ability to bind to common Smad4 protein after TGFβ activation in Mv1Lu cells has already been demonstrated.

In Figure 4-5, endogenous km23-1 was observed to be present in a complex with PKA RIβ in a TGFβ-dependent manner. This was observed as early as 5 min (top panel, lane 2), with a decrease at 30 min (top panel, lane 4). This time course is similar to what has been seen for TGFβ-induced PKA-Smad4 interaction, suggesting that a multi-protein complex might be formed, which includes km23-1, Smads, and PKA, in response of TβR activation. km23-1 is proposed to function as a motor receptor, which carries this proteins complex along the MTs through the binding to DIC.

Figure 4-3 has been shown that PKA phosphorylates km23-1 at 30 min after TGFβ treatment. In the same cell line, Figure 4-5 has been shown that the interaction between km23-1 and the regulatory subunit of PKA starts to decrease at 30 min after TGFβ treatment. The emerging picture from these results is that the phosphorylation at 30 min leads to a dissociation of the signaling protein complex.
Figure 4.5 km23-1 interacts with PKA regulatory subunit in a TGFβ-dependent manner

Mv1Lu cells were treated with TGFβ-1 (10 ng/ml) for 0, 5, 15, 30 min. Cell lysates (500 µg) were used for IP with 1 µg of anti-PKA RIβ subunit Ab, and then blotted with km23-1 Ab (pkm23 27-43) (top panel). The same membrane was then blotted with the PKA RIβ subunit Ab to show equal IP’d proteins (middle panel, lane 1-4). Lane 6 is the negative control for the IP Ab in which an RIβ blocking peptide (b.p.) was added to the cell lysates during the IP incubation. Lower panel, equal expression of endogenous km23-1 protein by IP/blot analysis using the km23-1 Ab against 1-15 as the IP Ab, and the km23-1 Ab against 27-43 as the blotting Ab.
4.6 The S73A mutant of km23-1 inhibits TGFβ-dependent transcriptional activation of the ARE-lux reporter.

As mentioned above, we have demonstrated that PKA could phosphorylate km23-1 at S73 after TGFβ activation, and that the PKA inhibitor H89 could inhibit km23-1 phosphorylation, as well as DIC binding. We have also shown that the phosphorylation mutant S73A of km23-1 could not interact with DIC or with wt km23-1. Base upon our model for km23 function, it was conceivable that the S73 mutant of km23-1 could disrupt downstream TGFβ signaling due to deficiency in DIC binding.

The ARE-lux reporter was previously shown to be activated by TGFβ or activin in a Smad2-dependent manner. Our lab has previously shown that transcriptional activation of the ARE-lux reporter could be inhibited by either siRNA-km23-1, or by an exon-3-truncated mutant of km23. Therefore, to assess whether S73 phosphorylation was required for TGFβ signaling, we performed ARE-lux reporter assays in Mv1Lu cells using S73-km23 mutant.

We transiently transfected Mv1Lu cells with either wt-km23-1 or S73-km23-1, along with the ARE-lux reporter and the co-activator FAST-1. We then performed ARE-lux luciferase reporter assays in the absence or presence of TGFβ. As shown in Figure 4.7, TGFβ induced ARE-lux activity in the EV and wt-km23-1 cells. In contrast, S73A mutant resulted in a significant repression of the ability of TGFβ to induce ARE reporter activity (from 9.4-fold for wt-km23 to 2.6-fold for S73A-km23). Thus, our results show that phosphorylation on Serine 73 of km23-1 is required for TGFβ-dependent regulation.
of the ARE promoter. In addition, base levels of ARE-lux were increased by S73A-km23-1, suggesting aberrant regulation of this promoter in the absence of TGFβ.
Figure 4.6 Serine 73 is required for TGFβ induction of Smad2-dependent transcriptional activation in ARE-lux reporter assays.

Mv1Lu cells were transfected with the indicated amounts of either wt-km23-1 or S73A-km23-1, along with the ARE-lux reporter and FAST-1. Twenty four hrs after transfection, the medium was replaced with serum-free medium for 1 h, and the cells were incubated in the absence (open bars) or presence (black bars) of TGFβ1 (5ng/ml) for an additional 18 hrs. The fold induction of Luciferase activity by TGFβ is indicated in parentheses on top of the relevant bars. Error bars represent the standard error of the mean. The results are representative of at least two experiments, each performed in triplicate.
Chapter 5
Discussion

km23-1 was previously identified as both a novel TβR-interacting protein and a light chain of cytoplasmic dynein in humans. It is ubiquitously expressed and highly conserved. Our lab showed that km23-1 is phosphorylated and recruited to the dynein motor complex through binding to DIC in response of TGFβ signaling. Blockade of km23-1 is known to inhibit specific TGFβ downstream effects. Mutant versions of km23-1 detected in human ovarian cancer were shown to result in a defect in dynein complex formation, as well as aberrant TGFβ-dependent transcriptional regulation.

Recent results in our lab also provided the first evidence that km23-1 is required for Smad 2-translocation to the nucleus and subsequent transcriptional activation of Smad 2 target genes. Overall, these previous results suggested that the km23-DLC functions as a motor receptor for the intracellular transport of TGFβ components after TβR activation.

Based upon these previous results, we questioned how km23-1 is regulated to mediate its functions both as part of the dynein complex and as an intermediate in TGFβ signaling. We hypothesized that site-specific phosphorylation might be the underlying mechanism for regulation of km23-1 activity, since phosphorylation events are transient and reversible, and represent common regulatory events in signaling pathways. In addition, our lab previously demonstrated that km23-1 could be phosphorylated after...
TGFβ activation on serine residues, which is consistent with the kinase specificity of the TβRs being phosphorylated on Serine/Threonine residues.

Here, we demonstrate for the first time that multiple, site-specific phosphorylation of km23-1 is required for regulation of dynein functions in TGFβ signaling. To test our hypothesis, we generated S→A phosphorylation mutants of km23-1 on conserved serines, and examined their effects on km23-1 phosphorylation and DIC binding activity after TβRs activation. We showed that both S32 and S73 are critical phosphorylation sites. Interestingly, we also demonstrated that PKA kinase activity was required for km23-1 functions, and that PKA could directly phosphorylate km23-1 on S73. This is consistent with our phosphorylation site predictions from Prosite and NetPhos, since S73, but not S32, is located in one of the PKA consensus sites in the km23-1 sequence. Further, we report here that km23-1 and PKA regulatory subunits are present in the same complex after TGFβ treatment. In addition, the S73A mutant that could not be phosphorylated by PKA resulted in aberrant TGFβ-dependent ARE–Lux transcriptional activity. Since S73 is phosphorylated by PKA, we think that the other critical site S32 is probably phosphorylated directly by TβR II. Collectively, our results demonstrate for the first time that phosphorylation of the dynein light chain km23-1 is required for dynein complex formation and activation of downstream TGFβ signaling events.

Phosphorylation of other DLCs also plays roles in the regulation of various signaling pathways. For example, Tctex-1 (DYNLT) interacts with, and is phosphorylated by the cytoplasmic domain of BMPRII, a member of the TGFβ superfamily. A mutation of Tctex-1 that results in a defect in its phosphorylation
contributes to the pathogenesis of primary pulmonary hypertension. Tctex-1 can also be phosphorylated by PKC, and it plays a role in actin remodeling during neurite outgrowth. DLC-8 (DYNLL1), also known as DLC1, was identified to be phosphorylated at serine 88 by p21-activated kinase 1 (Pak1). This phosphorylation is required for Pak1-DLC1 interaction and cell survival regulation. Overexpression of DLC1, but not of a S88A phosphorylation mutant of DLC8, promotes a cancerous phenotype in breast cancer cells.

While this is the first report of a positive role of PKA in km23-1-dependent intracellular transport of TGFβ signaling components, several studies regarding PKA have revealed its roles in TGFβ signaling. A bioinformatics study identified a TGFβ receptor binding domain in the sequence of a Caenorhabditis elegans AKAP, a specific adaptor protein for PKA. It suggested that the PKA holoenzyme is probably targeted to an AKAP complex, which includes the hetero-tetramer of TβRs. Another study has reported that PKA is activated and co-localized with Smads proteins at 15-30 min after TGFβ treatment, and that this association is required for specific downstream TGFβ-dependent responses. Our results in the same cell line showed that km23-1 is also co-localized with PKA during this time period, suggesting that a multi-protein complex may be formed after TGFβ ligand binding. This complex may include the TβRs, Smads, PKA, and km23-1. km23-1 may function as a receptor carrying this multi-protein complex to DIC, thereby initiating the retrograde transport of the signaling components along MTs.
It is well known that PKA forms a complex with molecular motors, such as dynein and kinesin, on organelles and that it regulates intracellular transport. It plays a critical role in dynein complex integrity, since removal of PKA causes dissociation of dynein and disruption of dynein-dependent intracellular transport of pigment granules. PKA also phosphorylates p150<sup>Glued</sup>, a subunit of the dynactin complex that plays a critical role in both cargo binding and dynein-mediated transport. Based upon previous reports and our results, PKA activity may be required for phosphorylation of p150<sup>Glued</sup>, or for structural support of the dynein complex at early times after TGFβ addition. In contrast, PKA phosphorylation of km23-1 at later times after TGFβ treatment (ie 30 min) in Mv1Lu cells may result in dissociation of the complex containing km23-1, DIC, PKA, and Smads. Thereafter, the PKA catalytic subunits may be released from the holoenzyme and move into nucleus. This is consistent with previous demonstrations that TGFβ stimulates translocation of the catalytic subunit of PKA to the nucleus for specific gene transcription regulation at 30 min after TGFβ treatment.

Here we propose a model for the role of km23-1 phosphorylation in dynein regulation of TGFβ signaling. Within minutes of ligand binding, km23-1 interacts with the activated TGFβ receptors, which are internalized into EEA1-enriched endosomes. Smad2 and PKA are also recruited to the EEA1-enriched endosomes through the TβR-binding domains and through the FYVE domains of both SARA and AKAP. Once km23-1 is phosphorylated by TGFβ RII, probably on Serine 32, DIC is recruited to this multi-protein complex and initiates km23/dynein-mediated transport of TGFβ signaling endosomes along MTs. In this regard, our previous results have shown that the
interaction of km23-1 with DIC is dependent upon kinase-active RII, which is consistent with our data that phosphorylation of km23-1 occurs on Serine 32, and is required for this interaction. At early times after ligand binding, PKA activates other components of the dynein complex, such as p150Glued and promotes dynein-mediated retrograde transport. As late as 30 min after TGFβ treatment, PKA activation that is induced by TGFβ remains very high \cite{71,72}. PKA phosphorylates km23-1 on serine 73 at approximately 30 min after TGFβ treatment, at which time the PKA regulatory subunit starts to dissociate from km23-1. By this time, Smad proteins would have translocated into the nucleus, suggesting that the PKA phosphorylation event may be very important for the negative regulation of the km23/dynein/MT complex. The dissociation of this complex may then affect the recycling of km23-1 and DIC into the cytoplasm, and the translocation of the catalytic subunit of PKA to the nucleus. This model may represent an efficient way to switch between different modes of regulation and to fine-tune this balance inside the cell. Along these lines, the S73A mutant of km23-1 may inhibit TGFβ-dependent transcriptional activation because it blocks PKA regulation of km23-1 phosphorylation, and the associated dynein complex dynamics.

Phosphorylation of the other two DLCs also modulates their functions. It was reported that phosphorylation of LC8 and Tctex-1 by Pak1 and PKC, respectively, promotes dissociation from DIC \cite{105}. Biochemical and NMR analyses revealed that different mechanisms were employed by these two DLCs. Phosphorylation of Tctex-1 directly masked the DIC binding site, whereas phosphorylation of LC8 dissociated the dimer and indirectly eliminated the DIC binding site \cite{105}. In the case of km23-1, its three-dimensional solution structure shows that S73 is lying on the putative DIC binding
surface that includes the two N-terminal α-helices. It suggests that S73 phosphorylation of km23-1 may directly abolish its DIC binding surface like for Tctex-1.

It is known that PKA activation is required for TGFβ stimulation of fibronectin expression \(^71\), and that blockade of km23-1 also significantly decreased induction of fibronectin expression. In the future, it would be of interest to investigate whether phosphorylation of km23-1 by PKA modulates this key TGFβ response.

Previous study, which applied laser-capture microdissection and nested reverse-transcription-PCR, has identified that km23-1 is altered in 42% human ovarian cancer patient tissues \(^32\). Such a high frequency rate suggests that alteration in km23-1 might play an important role in ovarian cancer formation or progression, probably through a mechanism involve a disruption of TGFβ components intracellular trafficking. Here, we demonstrated that inducible phosphorylations of km23-1 are critical to its “motor receptor” functions. Mutations at critical phosphorylation sites of km23-1 can block its interaction with DIC and inhibit downstream TGFβ-dependent transcription activation. Thus, it would be interesting in the future to examine the effects of mutating km23-1 at key phosphorylation sites, on the malignant phenotype of the human cancer cells in vitro, and on tumor progression in vivo.

Fibronectin, a major component of the extracellular matrix (ECM), has been implicated in carcinoma development \(^106\). TGFβ is one of the most potent stimulators of the ECM, and has been shown to potently induce fibronectin expression at both the
mRNA and protein levels\textsuperscript{107, 108}. Fibronectin gene stimulation could also be controlled by PKA stimulation via CRE sites\textsuperscript{109}. In addition, previous study has shown that inhibition of PKA in mesangial cells by overexpressing PKI attenuates TGFβ-mediated stimulation of fibronectin mRNA levels, suggesting that PKA activation may be required for TGFβ stimulation of fibronectin expression\textsuperscript{71}. Interestingly, our lab has demonstrated that blockade of km23-1 using siRNAs also significantly decreased TGFβ induction of fibronectin expression\textsuperscript{29}. Considering the results we are showing here that km23-1 is phosphorylated by PKA after TβR activation, it would be of interest in the future to investigate whether phosphorylation of km23-1 by PKA modulates TGFβ-stimulated fibronectin expression, which is a key TGFβ response implicated in human tumor development and progression.


86. Dean DC, Newby RF, Bourgeois S. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor beta, and cAMP in human cell lines. *J Cell Biol.* 1988;106:2159-2170.


