Published in final edited form as:

Exp Cell Res. 2013 April 1; 319(6): 897–907. doi:10.1016/j.yexcr.2012.12.029.

Requirement for protein kinase A in the phosphorylation of the TGF β receptor-interacting protein km23-1 as a component of TGF β downstream effects

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Abstract

km23-1 was previously identified as a TGF β -receptor interacting protein that was phosphorylated on serines after TGF β stimulation. In the current report, we examined the role of km23-1 phosphorylation in the downstream effects of TGF β / protein kinase A (PKA) signaling. Using phosphorylation site prediction software, we found that km23-1 has two potential PKA consensus phosphorylation sites. In vitro kinase assays further demonstrated that PKA directly phosphorylates km23-1 on serine 73 (S73). Moreover, our results show that the PKA-specific inhibitor H89 diminishes phosphorylation of km23-1 on S73 after TGF β stimulation. Taken together, our results demonstrate that TGF β induction of PKA activity results in phosphorylation of km23-1 on S73. In order to assess the mechanisms underlying PKA phosphorylation of km23-1 on S73 (S73-km23-1) after TGF β stimulation, immunoprecipitation (IP)/blot analyses were performed, which demonstrate that TGF β regulates complex formation between the PKA regulatory subunit RI β and km23-1 in vivo. In addition, an S73A mutant of km23-1 (S73A-km23-1), which could not be phosphorylated by PKA, inhibited TGF β induction of the km23-1-dynein complex and transcriptional activation of the activin-responsive element (ARE). Furthermore, our results show that km23-1 is required for cAMP-responsive element (CRE) transcriptional activation by TGF β , with S73-km23-1 being required for the CRE-dependent TGF β stimulation of fibronectin (FN) transcription. Collectively, our results demonstrate for the first time that TGF β /PKA phosphorylation of km23-1 on S73 is required for ARE- and CRE-mediated downstream events that include FN induction.

Keywords

TGF β ; km23-1; protein kinase A; phosphorylation; signal transduction

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Conflict of Interest Statement: The authors have no conflicts of interests to declare with the work presented herein.

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Introduction

The transforming growth factor (TGF β) superfamily is a large family of structurally related cell regulatory proteins that are differentially expressed and regulates diverse cellular processes, including cell proliferation, differentiation, apoptosis, fibrosis, wound repair, and inflammation in a wide range of target cells (Kang et al., 2009; Mu et al., 2012; Padua and Massague, 2009; Yue and Mulder, 2001). To initiate signaling, TGF β binds to and brings together type I (T β RI) and type II (T β RII) TGF β serine/threonine kinase receptors on the cell surface, followed by transphosphorylation of T β RI. The activated receptor complex stimulates intracellular mediators of TGF β signaling, including receptor-activated Smads (RSmads). These activated RSmads can form a complex with the common partner Smad4 and translocate to the nucleus, where they regulate transcription of a wide range of target genes (Kang et al., 2009; Padua and Massague, 2009). Besides the Smad-dependent pathway, TGF β signaling involves the activation of a number of other signaling pathways, including Ras/mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K)/ AKT, and PKA (Kang et al., 2009; Mu et al., 2012; Mulder, 2000; Padua and Massague, 2009; Yue and Mulder, 2001; Zhang, 2009).

PKA is a cytosolic, tetrameric holoenzyme that is composed of two regulatory subunits associated with two catalytic subunits in its inactive state (Taylor et al., 1990; Zhang et al., 2012). PKA signaling has been shown to play an important role in multiple physiological processes, including growth, differentiation, extracellular matrix production, cell motility, and apoptosis (Howe, 2011; Kotani, 2012; Walsh and Van Patten, 1994). TGF β also regulates many of these cellular processes, and there are several lines of evidence demonstrating interactions between TGF β signaling pathways and PKA. For example, previous results have shown that TGF β stimulates PKA signaling events in both mesangial and mink lung epithelial (Mv1Lu) cells that involve TGF β stimulation of cAMP response element-binding protein (CREB) phosphorylation and fibronectin (FN) induction (Wang et al., 1998; Zhang et al., 2004). In addition, TGF β induction of a Smad3/Smad4 complex has been shown to directly activate PKA independent of increased cAMP in Mv1Lu cells (Zhang et al., 2004). However, the molecular mechanisms underlying the interaction of TGF β and PKA signaling pathways are still not fully understood.

We have previously identified km23-1 as a TGF β receptor-interacting protein in a novel screen (Tang et al., 2002). km23-1, also termed km23 (Campbell et al., 2006; Kang et al., 2007; Tang et al., 2002), Robl1 (Nikulina et al., 2004), DNLC2A (Jiang et al., 2001), mLC7-1 (Tang et al., 2002), DYNLRB1, (Pfister et al., 2005) has been shown to interact with Rab6 (Wanschers et al., 2008), the human reduced folate carrier (Ashokkumar et al., 2009), dynein intermediate chain (DIC) (Susalka et al., 2002; Tang et al., 2002), and LIN-5 and GPR-1/2 in *C. elegans* (Couwenbergs et al., 2007). We have shown that km23-1 undergoes rapid phosphorylation on serine residues after T β R activation, in keeping with the kinase specificity of the T β Rs (Tang et al., 2002). Moreover, overexpression of km23-1 in TGF β -responsive Mv1Lu cells induced specific TGF β responses, such as Jun N-terminal kinase (JNK) activation and c-Jun phosphorylation (Jin et al., 2005). Furthermore, blockade of km23-1 decreased cellular responses to TGF β , including induction of FN expression and inhibition of cell cycle progression (Jin et al., 2005). Additional studies demonstrated that km23-1 can be a signaling mediator for Smad2-dependent TGF β signaling in mammalian cells (Jin et al., 2007). However, the mechanisms underlying km23-1's regulatory role in these major TGF β events is unclear. In the current report, we examined whether PKA could phosphorylate km23-1 in response to TGF β , and further, whether this phosphorylation could regulate specific TGF β /PKA pathway targets such as CRE and FN transcription.

Materials and Methods

Reagents

TGF β 1 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). The mouse IgG was from Sigma-Aldrich, and the rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-DIC monoclonal antibody (Ab) (MAB1618) was from Chemicon (Temecula, CA). The anti-FLAG M2 (F3165) Ab was from Sigma-Aldrich (St. Louis, MO). γ -[32 P]ATP (BLU002H) was from PerkinElmer Life Sciences (Boston, MA). The PKA inhibitor H89 was from Calbiochem. The anti-PKA RI β Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit hkm23-1 (1-15) and hkm23-1(27-43)w Abs were described previously (Jin et al., 2007; Jin et al., 2005). Pathdetect CRE-Luc cis-reporter plasmid was from Stratagene (La Jolla, CA, USA).

Constructs

The Flag-tagged serine mutants of km23-1, S32A-km23-1 and S73A-km23-1, were produced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) from Flag-tagged km23-1 (Tang et al., 2002) following the manufacturer's protocols. His-tagged S32A-km23-1 and S73A-km23-1 constructs were produced using the QuickChange Site-Directed Mutagenesis Kit from His-tagged km23-1 (Ilango et al., 2005). The Flag-tagged Δ S32A-km23-1 construct was produced using the QuickChange Site-Directed Mutagenesis Kit from Flag-tagged Δ km23-1 (Jin et al., 2007). The V5-tagged RI construct was generated by inserting the Alk-5 RI cDNA fragment (provided by Dr. K. Miyazono), 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 Lunenfeld Res. Institute, Toronto, Canada). All constructs were verified by DNA sequencing in both directions.

Cell culture

Mv1Lu (CCL-64) and 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were grown in Dulbecco's Modified Eagle's Medium with 10% (v/v) heat-inactivated fetal bovine serum. Cells were maintained in 5% CO $_2$ at 37°C. Cultures were routinely screened for mycoplasma using Hoechst 33258 staining.

Antibody production

The anti-serine-73 phospho-specific Ab (S73-P-km23-1) was custom-generated in rabbits by Anaspec (San Jose, CA), prepared against the phosphospecific serine-73 peptide (TFLRIR (pS) KKNE-NH $_2$) conjugated to keyhole limpet hemocyanin (KLH). The S73-P-km23-1 was further purified by positive-selection affinity chromatography using the phosphopeptides and by negative-depletion using nonphosphopeptides (TFLRIR S KKNE-NH $_2$). Transient transfections, in vivo phosphorylation assays, immunoprecipitation (IP)/blot, and Westerns were performed essentially as described previously (Jin et al., 2007; Jin et al., 2005; Jin et al., 2009; Tang et al., 2002).

In vitro kinase assays

To measure in vitro km23-1 phosphorylation by PKA, 12 μ g of purified His-km23-1 fusion protein and 10 μ Ci of γ - 32 P ATP (from PerkinElmer) were added to buffer provided by a PKA assay kit (Upstate). For PKA assays, three different concentrations (10 ng, 20 ng, or 40 ng) of the catalytic subunit of PKA (Upstate) were examined. 5 μ M of PKA inhibitory

peptide (PKI) (Bachem) was included in one reaction with 20 ng of PKA as a negative control. Kinase reactions were conducted at 30°C for 30 min and terminated by the addition of SDS-PAGE sample buffer. The proteins were subjected to 15% SDS-PAGE and ³²P-labelled fusion proteins were detected by autoradiography.

Luciferase reporter assays

For ARE-Lux assays, Mv1Lu cells were co-transfected with ARE-Lux and FAST-1 (Felicci et al., 2003; Jin et al., 2007; Piek et al., 2001; Yeo et al., 1999). TGFβ-dependent SBE₂-Luc, CRE-Luc, and pFN-Luc reporter assays were performed as described previously (Jin et al., 2007) using either FuGene 6 (Roche, Cat# 11814443001) or Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Renilla was used to normalize transfection efficiencies. 24 h after transfection, the cells were washed once with serum-free (SF) medium and incubated in SF medium for 1 h prior to TGFβ1 (10 ng/ml) treatment for an additional 18 h. Luciferase activity was measured using Promega's Dual-luciferase Reporter Assay System following the manufacturer's instructions. All assays were performed in triplicate.

Statistical analyses were by Student's t test. Triplicate samples were analyzed and mean ± SE plotted unless otherwise indicated.

Results

Our previous results have shown that km23-1 is a TGFβ signaling component that associates with the activated TGFβ receptor (TβR) complex endogenously, and is phosphorylated on serine residues (Tang et al., 2002). In order to identify other putative kinases that may phosphorylate km23-1, besides the TβR kinase, we used NetPhosK 1.0 to predict the potential phosphorylation sites in the km23-1 sequence. Previous studies have shown that for a given specificity level, NetPhosK-PKA achieves a higher sensitivity rate compare to other prediction methods (Blom et al., 2004). Using NetPhosK1.0, two potential PKA consensus phosphorylation sites were identified in the km23-1 sequence. One of the PKA consensus sites covers serine 13 (S13) (0.52, with the maximum of 1) and the other starts with serine 73 (S73) (0.76, with the maximum of 1). The maximum values indicate best overall prediction performance (Blom et al., 2004). Thus, the prediction results suggest that PKA might be another potential kinase for km23-1 besides the TβRs.

In order to determine whether 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-km23-1 protein as the substrate. As shown in Fig. 1A, PKA directly phosphorylated km23-1 in vitro (lane 2). A PKA inhibitory peptide completely blocked the phosphorylation of km23-1 by PKA (lane 3). No signal was detected in the absence of both PKA and PKA inhibitory peptide (lane 1). Similarly, the top panel of Fig. 1B further shows the direct phosphorylation of km23-1 by PKA in a dose-dependent manner (top panel, lanes 1-3). As expected, the PKA inhibitory peptide (5 μM) completely blocked the phosphorylation of km23-1 by PKA (top panel, lane 4). Lower panels show Coomassie blue staining of the expression of His-km23-1 fusion proteins and equal loading of the PKA catalytic subunit. Thus, our results demonstrate that PKA is able to directly phosphorylate km23-1 in vitro. Our previous results and those of others have shown that both S13 and S73 are conserved and are located on the surface (Hall et al., 2010; Ilangovan et al., 2005), which suggested that they might play important roles in protein function. Since the prediction results indicated that S73 scored higher than S13, we hypothesized that S73 was the major PKA phosphorylation site in km23-1.

In order to determine whether PKA could directly phosphorylate km23-1 at S73, we generated a S73 phospho-specific Ab (S73-P-km23-1) against the phospho-S73 peptide

(TFLRIR (pS) KKNE-NH₂). Furthermore, we performed *in vitro* kinase assays as described for Fig. 1B, and then blotted the membrane with the S73 phospho-specific anti-km23-1 Ab (S73-P-km23-1). As shown in Fig. 1C, phosphorylation detected by the S73-P-km23-1 Ab is also dependent upon the dose of PKA. It is clear that the level of S73-km23-1 phosphorylation was increased with the addition of the PKA catalytic subunit over the concentration range of 0.01 - 0.04 μ g (lanes 1-3, top panel). Collectively, our results demonstrate that PKA directly phosphorylates km23-1 on S73.

Previous studies have shown that PKA plays important roles in TGF β signaling (Wang et al., 1998; Yang et al., 2008). In addition, our results have shown that km23-1 is directly phosphorylated by PKA on S73 *in vitro*. Therefore, it was of interest to determine whether PKA could phosphorylate km23-1 on S73 in response to TGF β receptor activation in Mv1Lu cells. Accordingly, we performed IP/blot analyses using a Flag Ab as the IP Ab and an anti-S73 phospho-km23-1 Ab as the blotting Ab, in the absence and presence of TGF β . As shown in Fig. 2A, TGF β treatment affected the phosphorylation of km23-1, in contrast to no detectable phosphorylation in the absence of TGF β . Our results in Fig. 2B further confirmed that endogenous km23-1 is phosphorylated on S73 after TGF β treatment. To determine whether TGF β induction of km23-1 phosphorylation on S73 was PKA-specific, the cells were treated with the PKA-specific inhibitor H89 (Fig. 2C). H89 has been used in recent reports from other laboratories to implicate PKA in various signaling events (Maymo et al., 2012; Park et al., 2012). While phosphorylated km23-1-Flag was detectable with the S73-P-km23-1 Ab by 30 min after TGF β treatment (top panel, lane 2), no corresponding band was observed with H89 treatment (top panel, lane 3). Collectively, these findings demonstrate that PKA phosphorylates km23-1 on S73 *in vivo* in response to TGF β .

Regulation by PKA usually involves localization of the PKA regulatory complex in direct proximity to its substrates. Since our results have shown that PKA directly phosphorylates km23-1 on S73 in response to TGF β , it was of interest to determine whether km23-1 interacts with the PKA regulatory subunit in the absence and presence of TGF β . Accordingly, we performed IP/blot analyses using an Ab against the RI β regulatory subunit of PKA, and blotted with a rabbit anti human km23-1 [hkm23-1(27-43)w] Ab. The RI β isoform was used since it was previously shown to bind Smads following TGF β activation in Mv1Lu cells (Zhang et al., 2004). As shown in Fig. 3, endogenous km23-1 was observed to be present in a complex with PKA RI β in a TGF β -dependent manner, as early as 5 min after TGF β (top panel, lane 2), with a decrease at 30 min (top panel, lane 4). This time course is similar to that for the TGF β -induced PKA-Smad interaction (5-30 min) (Yang et al., 2008; Zhang et al., 2004), suggesting that the formation of the complex between km23-1 and PKA RI β occurs during the time when PKA is activated by TGF β .

Our previous results (Tang et al., 2002) have shown that TGF β induces the interaction between km23-1 and the DIC subunit of the dynein motor complex over a time frame similar to that for TGF β induction of the PKA-Smad complex (Zhang et al., 2004). Further, disruption of the dynein complex inhibits TGF β induction of both nuclear translocation of Smad2 and Smad2-dependent transcriptional activation (Jin et al., 2007). These findings suggest that the km23-1-dynein complex is important for downstream TGF β /Smad2 signaling. Consequently, here we examined whether an S73A mutant of km23-1 would disrupt the km23-1 interaction with DIC. For these studies, we performed IP/blot analyses in 293T cells using anti-DIC as the IP antibody, and anti-Flag as the blotting antibody, after transient expression of either wt-km23-1 or S73A-km23-1 together with the T β Rs. As shown in Fig. 4A, TGF β activation induced the recruitment of km23-1 to the DIC (lane 2), whereas the S73A-km23-1 mutant significantly blocked this interaction (lane 3). No specific bands were detectable in the EV (lane 1) and IgG (lane 4) control lanes. Other panels in Fig. 4A depict equal expression and loading for DIC, T β RII, and km23-1 proteins. In addition,

performing IP/blot analyses in the opposite direction revealed the same disruption of the km23-1-dynein complex by the S73A-km23-1 mutant (Fig. 4B). Thus, our results suggest that the S73 site in km23-1 is important for downstream TGF β /Smad2 signaling.

TGF β is known to activate the ARE-Lux reporter via complex formation of FAST-1 with Smad2 (Chen et al., 1997). Since we have previously shown that km23-1 knockdown reduced TGF β /Smad2 signaling (Jin et al., 2007), we tested the effect of the S73 mutant of km23-1 on Smad2-driven ARE-Lux reporter activity (Fig. 5A). km23-1, which by itself has no DNA binding or transactivation properties, did not significantly stimulate ARE-Lux beyond that detected for empty vector (EV). In contrast, the inactivating S73A mutant of km23-1 reduced the fold induction of ARE activity by TGF β . Interestingly, S73A-km23-1 also stimulated basal levels of ARE-Lux, and the increase was statistically significant ($p < 0.01$). In order to more clearly define the effects of the S73A-km23-1 mutant on both basal and TGF β -stimulated ARE-lux, we expressed the S73A-km23-1-Flag at increasing concentrations. As shown in Fig. 5B, S73A-km23-1 displayed a dose-dependent decrease in the fold induction of ARE-Lux activity by TGF β with increasing doses of S73A-km23-1-Flag. Further, basal ARE-lux activity was increased in a dose-dependent manner. Our results demonstrate that phosphorylation on S73 of km23-1 is required for TGF β -dependent regulation of the ARE promoter. In contrast, inactivation of km23-1 phosphorylation at S73 stimulated ligand-independent ARE-lux activation. Our findings are consistent with the contention that S73A-km23-1 relieves an autoinhibitory effect on the ARE, suggesting that autocrine regulation of S73-km23-1 normally represses Smad2/FAST-1/ARE activity in the absence of exogenous TGF β .

To determine whether S73A-km23-1 would also modulate TGF β /Smad3-dependent Smad binding element (SBE) promoter reporter activity, we performed similar luciferase reporter assays using the SBE₂-Luc previously described (Jin et al., 2007; Zawel et al., 1998). In this regard, the SBE₂-Luc reporter has been used previously to demonstrate Smad3/4-dependent responses induced by TGF β (Felicetti et al., 2003). As shown in Fig. 5C, S73A-km23-1 had no effect on TGF β -inducible SBE₂-Luc. Thus, our results demonstrate that phosphorylation on S73 of km23-1 is specifically required for TGF β -dependent regulation of the Smad2-dependent ARE, but not for Smad3-dependent SBE reporter activity.

It is known that PKA activation is required for TGF β stimulation of fibronectin expression (Singh et al., 2004; Wang et al., 1998), and that blockade of km23-1 also significantly decreased induction of fibronectin expression (Jin et al., 2005). Therefore, it was of interest to investigate whether phosphorylation of S73-km23-1 by PKA modulates this key TGF β response. Fibronectin gene stimulation by TGF β is considered to be controlled, at least in part, by CRE sites located in the fibronectin promoter (Dean et al., 1988). Therefore, we first examined whether TGF β regulation of a CRE reporter would be affected by km23-1 over-expression in the absence or presence of TGF β . As shown in Fig. 6A, co-expression of RII-HA with km23-1 resulted in an increase in CRE-Luc luciferase activity (closed bars, 3rd set), relative to that observed after expression of EV alone (1st set), or after co-expression of RI-Flag with either EV or km23-1 (2nd set), or after co-expression of RII-HA with EV (open bars, 3rd set). Since co-expression of both TBRs is known to result in heteromeric complex formation and receptor activation in the absence of ligand (Ventura et al., 1994), as expected, expression of both I and TBR II without km23-1 stimulated CRE-Luc luciferase activity (open bars, 4th set). More significantly, co-expression of km23-1 with both TGF β receptors strongly enhanced CRE-Luc reporter activity in 293T cells (closed bars, 4th set). Thus, our results demonstrate that km23-1 is required for CRE transactivation, which is enhanced by TBR activation. Similarly, we performed CRE-Luc luciferase reporter assays after transiently transfecting Mv1Lu cells (having endogenous TBRs) with either EV or km23-1-Flag in the absence and presence of TGF β . As shown in Fig. 6B, over-expression of

km23-1 resulted in a significant increase in trans-activation of the CRE-Luc in the absence and presence of TGF β . Thus, our results demonstrate that km23-1 can synergize with T β R activation and with TGF β to stimulate CRE luciferase reporter activity.

To further explore the functional significance of km23-1 in TGF β /PKA signaling downstream to FN, we performed pFN-Luc luciferase reporter assays after transient transfection with either S73 km23-1-Flag or the other indicated plasmids, along with the pFN-Luc reporter, in the absence or presence of TGF β . The pFN-Luc luciferase reporter has been described previously (Michaelson et al., 2002). As shown in Fig. 6C, TGF β induced pFN-Luc luciferase activity in the EV and km23-1 cells. In contrast, S73A-km23-1 resulted in a significant inhibition of TGF β induction of FN-Luc reporter activity, while S32A-km23-1 had no effect. As expected, siRNA depletion of km23-1 significantly decreased pFN-luc luciferase reporter activation. Thus, our results demonstrate that phosphorylation of km23-1 on S73 by PKA is required for TGF β induction of FN transcriptional activity, a major downstream response of both PKA and TGF β .

Discussion

In the current report, we demonstrate for the first time that PKA kinase activity was required for km23-1 functions involving the direct phosphorylation of S73-km23-1. This is consistent with predictions from NetPhos K whereby S73 is located within a PKA consensus site. The PKA and S73-km23-1 relationship was further characterized, and revealed that km23-1 and the PKA R1 β regulatory subunits are present in the same complex after TGF β treatment. The S73A mutant that could not be phosphorylated by PKA resulted in diminished TGF β -dependent regulation of both ARE and FN transcriptional activity. Collectively, our results demonstrate for the first time that phosphorylation of S73-km23-1 by PKA is required for activation of downstream TGF β signaling events.

Our findings also reveal a novel TGF β /PKA/km23-1/CRE effector pathway, mediated by km23-1 phosphorylation at S73, and leading to FN transcriptional activation. Along these lines, the mechanism(s) by which TGF β induces FN expression are still unclear. The FN promoter contains three CRE sites (Bowlus et al., 1991), one of which (CRE-170) has been shown to be occupied by c-Jun-ATF-2 (van Dam et al., 1993). It is also known that PKA activation is required for TGF β stimulation of FN gene expression, with both CREB and ATF-1 binding to the CRE site (Wang et al., 1998). Howe and co-workers (Hocevar et al., 1999; Hocevar et al., 2005) have reported that TGF β induction of FN expression requires the JNK pathway. We have shown that blockade of km23-1 significantly decreased induction of FN expression, demonstrating that km23-1 is also required for FN expression (Jin et al., 2005). Further, overexpression of km23-1 induced JNK activation (Tang et al., 2002), whereas km23-1 depletion reduced TGF β activation of JNK (Jin et al., 2012a). Altogether, the evidence suggests that the TGF β /km23-1 induction of FN also requires JNK activation.

In addition to the TGF β /PKA/km23-1/CRE pathway mediating FN induction, it is possible that the S73-P-km23-1 effects on Smad2/ARE contribute to FN induction. The FN promoter also contains an activator protein-2 (AP-2) consensus phosphorylation site (Kornblihtt et al., 1996). More recently, Smad2 has been shown to interact in a complex with AP-2 to regulate TGF β signaling activity (Koinuma et al., 2009). Further, CREB binding protein (P300/CBP), which is known to bind to Smad2 transcriptional complexes (Braganca et al., 2003; Janknecht et al., 1998), was shown to bind to AP-2 promoter regulatory sites to enhance transcription (Braganca et al., 2003). Since FN represents one of these AP-2-regulated genes, the S73-P-km23-1 regulation of Smad2/ARE may ultimately activate transcription from the AP-2 site in the FN promoter, mediated by AP-2/Smad2/p300CBP complexes. According to this model, PKA phosphorylation of km23-1 would permit cross-talk between

the JNK/Jun/CRE and Smad2/ARE/AP-2 effector pathways, providing tight control of FN transcription. Along these lines, TGF β /Smad2-dependent regulation of FN has been reported for other cell types, although the mechanism was not elucidated (Lai et al., 2006).

Our results also show that PKA phosphorylation of km23-1 on S73 was detectable at 30 min after TGF β addition, at a time when the PKA R1 β regulatory subunit starts to dissociate from km23-1. This is in keeping with previous reports showing that PKA is activated by TGF β as soon as 15 min after TGF β treatment, often regulated by a Smad3/4 complex (Wang et al., 1998; Zhang et al., 2004). Thereafter, the PKA catalytic subunits may be released from the holoenzyme and move into nucleus to regulate gene transcription (Wang et al., 1998; Zhang et al., 2004). With regard to Smad signaling, here we show that S73-km23-1 is required for TGF β /Smad2 transcriptional events, but not for TGF β /Smad3 SBE transactivation. This is in contrast to previous reports, which implicate Smad3, but not Smad2, as a major component of the TGF β -stimulated complex with the regulatory subunit of PKA (Chowdhury et al., 2011; Zhang et al., 2004). In addition, the PKA/S73-P-km23-1 regulation of Smad2/ARE transcription observed here (Fig. 5A) likely occurs downstream from the TGF β -regulated T β RII-km23-1-Smad2 complex that we have previously described (Jin et al., 2007). Moreover, the finding that the S73A-km23-1 mutant inhibited the km23-1-dynein complex, which is important for subsequent Smad2 nuclear translocation, suggests that the PKA phosphorylation of km23-1 at S73 occurs prior to TGF β -mediated nuclear translocation of Smad2. Consequently, our findings support a role for km23-1 at multiple levels in the regulation of these critical signaling complexes, further demonstrating the importance of unraveling each of the unique steps at which km23-1 can function.

While this is the first report of a positive regulatory role for PKA in km23-1-dependent TGF β signaling, several studies regarding PKA have revealed roles for this kinase in TGF β signaling. A bioinformatics study identified a T β R binding domain in the sequence for a specific adaptor protein of PKA, one of the A-kinase anchoring proteins (AKAPs). It was suggested that the PKA holoenzyme is probably targeted to an AKAP complex, which includes the hetero-tetramer of T β Rs (Herrgard et al., 2000). Another study has suggested that AKAPs are required for the subcellular localization of PKA and for its interaction with Smads (Zhang et al., 2004). A more recent study demonstrated that the specific AKAP149 is a novel TGF β /PKA signaling transduceome, which has a critical role in TGF β -mediated PKA activation (Chowdhury et al., 2011). Here we have found that PKA phosphorylation of S73-km23-1 can regulate both TGF β -dependent and independent events, further illustrating the importance of this signaling intermediate in modulating cell phenotype. Along these lines, we have recently shown that km23-1 plays a critical role in RhoA/actin-based cell migration (Jin et al., 2012b). Since PKA is known to regulate RhoA (Tkachenko et al., 2011), future studies will address the mechanisms linking km23-1's effects on these critical components.

In summary, here we show that km23-1 interacts with the R1 β regulatory subunit of PKA. It is phosphorylated both in vitro and in vivo by PKA at S73, with the S73A-km23-1 mutant resulting in a significant inhibition of TGF β induction of FN transcriptional activity. In addition, km23-1 can synergize with the TGF β receptor complex and with TGF β to stimulate CRE-Lux luciferase reporter activity. Collectively, our results demonstrate that phosphorylation of km23-1 on S73 by PKA is required for TGF β induction of FN transcription, which is a CRE-mediated physiological response of both PKA and TGF β . In addition, the S73 phosphorylation site in km23-1 regulates Smad2/ARE activity, which may also play a role in induction of FN expression. Overall, it is likely that km23-1 functions as a novel mediator of signaling cross-talk between the PKA, JNK/Jun/CRE, and Smad2 pathways in the induction of FN by TGF β .

Acknowledgments

We thank Dr. Scott E. Kern (Johns Hopkins Oncology Center, Baltimore, MD) for SBE2-Luc, and Dr. Malcolm Whitman (Harvard Medical School, Boston, MA) for the ARE-Lux and FAST-1. We also thank Dr. Kohei Miyazono (University of Tokyo, Tokyo, Japan) for the V5-tagged RI construct, and Dr. Jeffrey L. Wrana (Samuel Lunenfeld Res. Institute, Toronto, Canada for the HA-tagged RII construct).

Grant sponsor: National Institutes of Health to K. M. M.

Grant Numbers: CA090765, CA092889 and CA092889-08S1

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Highlights

km23-1 is phosphorylated by PKA at serine 73 both in vitro and in vivo

TGF β regulates km23-1 interaction with the R1 β regulatory subunit of PKA

km23-1 synergizes with the TGF β ligand-receptor complex to stimulate the CRE

PKA phosphorylation of km23-1 is required for TGF β induction of FN transcription

km23-1 mediates signaling cross-talk between PKA, JNK/ CRE, and Smad2 pathways

Fig. 1. PKA directly phosphorylates km23-1 on S73 in vitro

A: Purified His-km23-1 fusion proteins were used as substrates in an in vitro kinase reaction with the recombinant catalytic subunit of PKA in the presence of [γ -³²P] ATP. **B:** Similar studies were performed as for **A** except with increasing amounts of the recombinant catalytic subunit of PKA used. Equal loading of His-km23-1 fusion proteins and of the catalytic subunit of PKA were revealed by Coomassie blue staining in the lower panels. **C:** After pull-down using Ni-NTA agarose followed by SDS-PAGE as for **A**, the His-fusion proteins were detected by blotting with the S73 phospho-specific km23-1 Ab. Lower panel, Western blot analysis showing equal loading of His-km23-1 using km23-1 (27-43)w Ab. The results are representative of triplicate experiments.

Fig. 2. TGFβ induces PKA phosphorylation of km23-1 on S73 in vivo, which is inhibited by H89

A: Mv1Lu cells were transfected with km23-1-Flag. 24h later cells were changed to SF conditions for 1 h prior to TGFβ1 (10 ng/ml) treatment for 30 min. Cell lysates were subjected to IP using a Flag Ab, and blotted using the S73 phospho-specific anti-km23-1 antibody (S73-P-km23-1). Lower panel, Western blot analysis showing equal expression of km23-1-Flag using anti-Flag. The results are representative of triplicate experiments. **B:** Endogenous km23-1 was IP'd using hkm23-1 (1-15) Ab, and blotted using S73-P-km23-1. Lower panel, Western blot analysis showing equal expression of endogenous km23-1 using km23-1(27-43)w Ab. **C:** Mv1Lu cells were transfected with km23-1-Flag, and treated with TGFβ1 as for **A**. For lane 3, cells were treated with H89 for 30 min prior to TGFβ1 treatment for 30 min. Cell lysates were treated and analyzed as for **A**. Lane 4 is the IgG control. The results are representative of triplicate experiments.

Fig. 3. km23-1 interacts with the R1β regulatory subunit of PKA in a TGFβ1-dependent manner
Mv1Lu cells were washed once with SF medium and incubated in SF medium for 1 h prior to TGFβ1 (10 ng/ml) treatment for the indicated times. Cell lysates (500 μg) were used for IP with 1 μg of anti-PKA R1β subunit Ab, and were then blotted with an anti-hkm23-1 (27-43)w Ab (top panel). The same membrane was then blotted with the PKA R1β subunit Ab to show equal IP'd proteins (1st middle panel, lane 1-4). Lane 6 is the negative control for the IP Ab in which an R1β blocking peptide (b.p.) was added to the cell lysates during the IP incubation. 2nd middle panel, equal expression of endogenous km23-1 protein was demonstrated by IP/blot analysis using the hkm23-1(1-15) as the IP Ab, and the hkm23-1(27-43)w as the blotting Ab. Bottom panel, the blots were scanned and quantified by using Image J software. The results are representative of triplicate experiments.

Fig. 4. S73 is required for TGFβ1 induction of the km23-1-DIC complex

A: 293T cells were transiently co-transfected with the indicated plasmids. 24 h after transfection, cells were incubated in SF medium for 60 min before addition of TGFβ1 (10 ng/ml) for 0, or 15 min. Cell lysates were then IP'd with an anti-DIC Ab and were then blotted with an anti-Flag Ab (top panel). The same membrane was then blotted with the DIC Ab to show equal IP'd proteins (1st middle panel). Equal inputs and expression of RII-HA, and Flag-tagged proteins were verified by Western blotting (bottom two panels). **B:** The IP/blot analysis using the samples from A was performed in the opposite direction with Flag as the IP Ab. Results are representative of two experiments.

Fig. 5. S73-km23-1 inhibits TGFβ induction of TGFβ/Smad2-dependent transcription in ARE reporter assays, but has no effect on TGFβ/Smad3-dependent transcriptional activation
A: Mv1Lu cells were transfected with EV, km23-1-Flag, or S73A km23-1-Flag, along with 0.2 μg of ARE-Lux reporter and 0.2 μg of FAST-1. To normalize transfection efficiencies, 0.2 μg of Renilla was co-transfected as an internal control. Luciferase reporter assays were performed as described in “Materials and Methods.” Error bars represent the SEM. The results are representative of at least two experiments, each performed in triplicate. Asterisks indicate a statistically significant difference ($p < 0.01$) of the fold increase between the km23-1-Flag-transfected Mv1Lu cells and the EV-transfected in the absence of TGFβ. Double asterisks indicate a statistically significant difference ($p < 0.01$) in the fold changes for +/-TGFβ between km23-1-Flag-transfected Mv1Lu cells and S73A km23-1-Flag-transfected Mv1Lu cells. **B:** Mv1Lu cells were transfected with increasing amounts of S73A km23-1-Flag, along with 0.2 μg of ARE-Lux reporter and 0.2 μg of FAST-1. **C:** Mv1Lu cells were transfected with either EV, km23-1, or S73A-km23-1, along with 0.2 μg of SBE₂-Luc. The results are representative of three similar experiments, each performed in triplicate.

Fig. 6. km23-1 induces CRE transcriptional activation by TGF β , and S73-km23-1 is required for TGF β stimulation of CRE-dependent FN transcription

A: 293T cells were transiently transfected with 0.2 μ g CRE-Luc, 0.05 μ g Renilla, and 0.1 μ g of either RI-Flag, RII-HA and km23-1-Flag as indicated. 48h later, cells were lysed and the luciferase reporter assays were performed as described in “Materials and Methods.” Open bars, -km23-1-Flag; closed bars, +km23-1-Flag. The results are representative of triplicate experiments. * $p < 0.01$. **B:** Mv1Lu cells were transfected with 0.4 μ g of km23-1 or EV, 0.4 μ g of CRE-Luc and 0.08 μ g of Renilla. 21h later, cells were changed to SF conditions for 1h, followed by treatment with TGF β 1 (10 ng/ml) for 24h. Luciferase reporter assays were performed as for **A**. The results are representative of triplicate experiments. * $p < 0.01$. **C:** Mv1Lu cells were transfected with EV, km23-1 siRNA, km23-1, S32 km23-1-Flag or S73A-km23-1-Flag, along with 0.4 μ g of pFN-Luc, 0.1 μ g of Renilla. Luciferase reporter assays were performed as described in **A**. The results are representative of at least two experiments, each performed in triplicate. Asterisks indicate a statistically significant difference ($p < 0.001$) of the fold changes between +/-TGF β for km23-1 siRNA-transfected Mv1Lu cells compared to EV-transfected Mv1Lu cells. Double asterisks indicate a statistically significant difference ($p < 0.001$) of the fold changes comparing +/-TGF β between S73A km23-1-Flag-transfected Mv1Lu cells and EV-transfected Mv1Lu cells. The results are representative of at least two experiments, each performed in triplicate.