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A direct interaction between TGFβ activated kinase 1 and the TGFβ type II receptor: Implications for TGFβ signalling and cardiac hypertrophy

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Abstract

Objective: Transforming growth factor beta (TGFβ)-activated kinase 1 (TAK1) is a MAP kinase kinase kinase involved in numerous signalling pathways and is strongly implicated in cardiac hypertrophy and heart failure. TGFβ is also associated with hypertension and heart disease, and evidence suggests that TGFβ1 and TAK1 act together in a cardiac stress signalling pathway. Canonical TGFβ signalling is mediated through Smad transcription factors, but TGFβ can also rapidly activate TAK1. The activation of the Smad cascade is well characterised, but little is known about how TAK1 is activated in response to TGFβ, and no direct link between any MAPK kinase pathway and the TGFβ receptors has yet been established. Since TAK1 is activated by TGFβ within 1 min in cardiomyocytes, we hypothesised there might be a direct interaction between TAK1 and one of the TGFβ receptors.
Methods: We used a combination of in vitro binding assays and co-immunoprecipitation (IP) experiments to investigate whether TAK1 interacted with the type I (ALK1 or ALK5) or type II (TBRII) TGFβ receptors. Interactions between endogenous proteins were tested using mouse myoblast and rat cardiomyocyte cells.
Results: Immunoprecipitation and in vitro binding assays show that TAK1 binds directly to TBRII. Precipitation of endogenous TAK1 protein in rat cardiomyocytes shows that, in addition to a direct association with TBRII, it also interacts indirectly with ALK5.
Conclusions: We describe a novel and specific interaction between TAK1 and TBRII which, for the first time, directly links TAK1 to the TGFβ signalling cascade and potentially explains how TGFβ signalling in cardiomyocytes mediates a hypertrophic response.

Keywords: Angiotensin; Growth factors; Heart failure; Hypertrophy; MAP kinase

1. Introduction

The molecular pathways that couple increased haemodynamic load to myocardial hypertrophy, cardiomyocyte apoptosis and subsequent heart failure are complex and represent an important challenge in current cardiovascular medicine [1–4]. Recently, an important cytokine, transforming growth factor β1 (TGFβ1), has been shown to be pivotal in mediating the cardiac hypertrophic response induced by angiotensin II [5–8]. TGFβ1 expression is stimulated by Angiotensin II and it becomes highly expressed in hypertrophic myocardium, where it regulates a wide range of cellular functions including proliferation, differentiation, apoptosis and fibrosis [9,10].

To promote signalling, TGFβ first binds to the TGFβ type II receptor (TBRII) at the cell surface stimulating it to activate the TGFβ type I receptor (TBRI), which in turn phosphorylates downstream Smad proteins. These activated Smad complexes translocate to the nucleus where they regulate the transcription of TGFβ target genes [11,12]. In addition to the canonical TGFβ signalling pathway, TGFβ has been shown to rapidly activate TGFβ activated kinase 1 (TAK1), a MAP kinase kinase kinase [13]. In cardiomyocytes, TAK1 is activated within a minute of exposure

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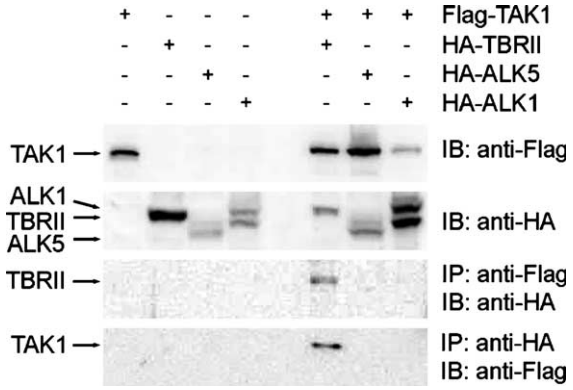

Fig. 1. TAK1 immunoprecipitates with the Type II TGFβ receptor. The two upper panels show immunoblot (IB) analysis of 293T cell lysates expressing Flag-TAK1 and HA-ALK1, HA-ALK5 (type I TGFβ receptors) or HA-TBRII (Type II TGFβ receptor), as indicated. Immunoprecipitation (IP) of TAK1 with anti-Flag antibody followed by immunoblot analysis with anti-HA antibody (3rd panel) detects TBRII but not ALK1 or ALK5. Reciprocal immunoprecipitation of the TGFβ receptors using anti-HA antibody, followed by immunoblot analysis with anti-Flag (bottom panel), detects TAK1 protein only in the TBRII precipitate.

to TGFβ1 [13,14] and there is clear evidence that TAK1 is also an important regulator of the hypertrophic response. Firstly, TAK1 is activated in cardiomyocytes following pressure overload generated by aortic constriction; secondly, cardiac specific over-expression of activated TAK1 in a transgenic mouse leads to cardiac hypertrophy and heart failure; and thirdly, dominant negative TAK1 protein can inhibit TGFβ-induced hypertrophic events in mouse cardiomyocytes and fibroblasts [14,15]. This particularly illustrates the importance of TAK1 as a mediator of cardiac TGFβ signalling, although TAK1 also interacts with the BMP and IL-1 signalling pathways and is arguably a central protein in the cellular signalling network [16–18]. Active TAK1 phosphorylates MKK3 and/or MKK6, which in turn phosphorylate p38. Increased p38 activity is strongly associated with typical hypertrophic responses including inflammation, fibroblast proliferation, hypertrophy and apoptosis [19–22].

The mechanism of intracellular TAK1 activation by extracellular TGFβ stimulation is not well understood.

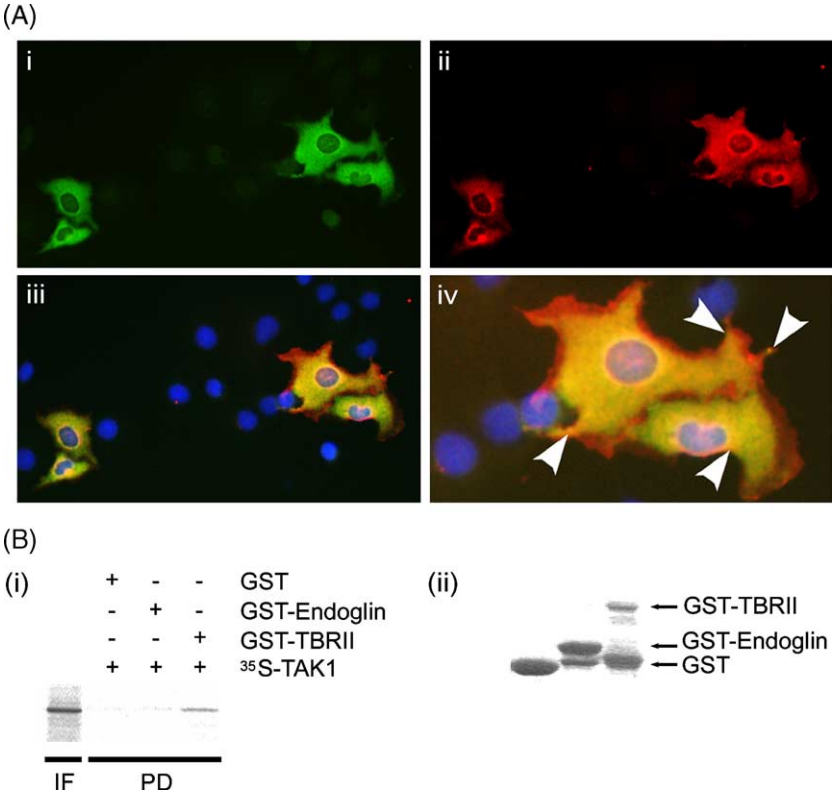

Fig. 2. (A) Cellular co-localisation of TAK1 and TBRII in Cos7 cells. Immunofluorescence microscopy of Cos7 cells co-expressing recombinant TAK1 and TBRII proteins. Cells were immunostained with (i) anti-TAK1 antibody and (ii) anti-TBRII antibody. The merged images (iii and iv) show areas of co-localisation in yellow (nuclei were detected with DAPI staining). Arrows indicate areas of co-localisation at the cell membrane. (B) In vitro binding assay with TAK1 and the cytoplasmic domain of TGFβ receptor proteins, TBRII and endoglin (a Type III TGFβ receptor). ^{35}S-labelled TAK1 protein was generated by in vitro transcription and translation and mixed with either glutathione S-transferase (GST) alone, GST–Endoglin fusion protein or GST–TBRII in an in vitro binding assay. (i) Autoradiograph shows that ^{35}S-TAK1 in the binding assay input fraction (IF) is seen after pull down (PD) of GST–TBRII, but not GST–Endoglin. (ii) Coomassie staining of GST, GST–Endoglin fusion protein and GST–TBRII fusion protein after SDS-PAGE, confirmed that GST and GST fusion proteins were present in the binding assays.

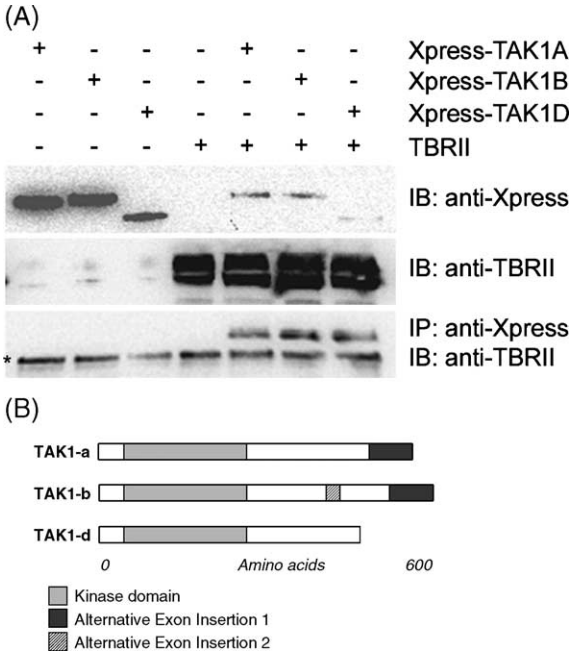

Fig. 3. Immunoprecipitation of TBRII with three different isoforms of TAK1. (A) Immunoblot analysis of Cos7 cell lysates expressing three TAK1 isoforms (A, B and D) and TBRII. TBRII is detected by immunoblotting after immunoprecipitation of all three TAK1 isoforms. (*)=Non-specific IgG band. (B) Diagrammatic representation of the three TAK1 isoforms used.

Early work showed that TAK1 is activated by TAK1-binding protein (TAB1), which is required for normal cardiac development [23,24], but this does not explain the rapid activation response to extracellular TGFβ. Here we describe a specific interaction between TAK1 and the type II TGFβ receptor (TBRII), which directly links TAK1 to the primary step of the TGFβ signalling cascade. This work is important not only as the first demonstration of a direct connection between a TGFβ receptor and TAK1 but also because of its potential importance in the early signalling events leading to cardiac hypertrophy and heart failure.

2. Methods

2.1. Plasmids and antibodies

HA-TAK1 and FLAG-TAK1 were cloned into pcDNA3.1^{+} (Invitrogen). The following plasmids were kind gifts: pCMV.HA-ALK1, pCMV.HA-ALK5, and pcDNA.HA-TBRII (P. ten Dijke), pCMV.myc-TAB1 (T. Ishitani), pGEX4T.mTBRII (P. Eyers), pXpressTAK1A, pXpressTAK1B and pXpressTAK1D (F. Guesdon), pcDL.HA-MLTKβ (E. Nishida). TAK1 was cloned into vector pFTX9 [25] to generate pFTX9.mTAK1 for in vitro transcription/translation. pGEX2T.mEndoglin GST-fusion plasmid was generated by PCR cloning of Endoglin cytoplasmic region cDNA into pGEX2T (Pharmacia). Primary antibodies were anti-TAK1 (sc-7967), anti-TBRII (sc-220) and anti-ALK5 (sc-398) antibodies (Santa Cruz), anti-Myc antibody (Oncogene Research Products), 3F10 anti-HA antibody (Roche), M2 anti-Flag antibody (Sigma) and anti-Xpress antibody (Invitrogen). Secondary antibodies were conjugated to HRP (DAKO) or FITC/Texas Red (Vector Laboratories).

2.2. Cell culture and transient transfections

Cos7 (African green monkey kidney) and 293T (Human embryonal kidney) cells were used for transfection studies and were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS) and 4 mM glutamine. Cells were transfected with 1 μg of plasmid using FuGene6 (Roche) and incubated for 24 to 48 h before appropriate treatment.

To examine endogenous protein interactions, C2C12 (mouse muscle myoblast) cells were cultured in DMEM supplemented with 15% FCS and 4 mM glutamine, whilst H9C2 (rat cardiomyocyte) cells were cultured in high glucose (4500 mg/ml) DMEM supplemented with 10% FCS and 4 mM glutamine.

2.3. Immunofluorescence microscopy

Cos7 cells were co-transfected with pcDNA3.1+FLAG-TAK1 and pcDNA.HA-TBRII plasmids and fixed in 100% methanol after 24 h. Cells were permeabilised in PBS/0.5% Triton X-100 for 30 min at 4 °C, blocked in PBS/0.1% Tween-20/10% FCS for 30 min at 37 °C and treated with anti-TAK1 antibody for 1 h at 37 °C before washing in PBS/0.1% Tween-20 and incubation with FITC-conjugated secondary antibody for 1 h (37 °C). For double staining, cells were washed thoroughly again and incubated with goat polyclonal anti-TBRII primary antibody, followed by Texas

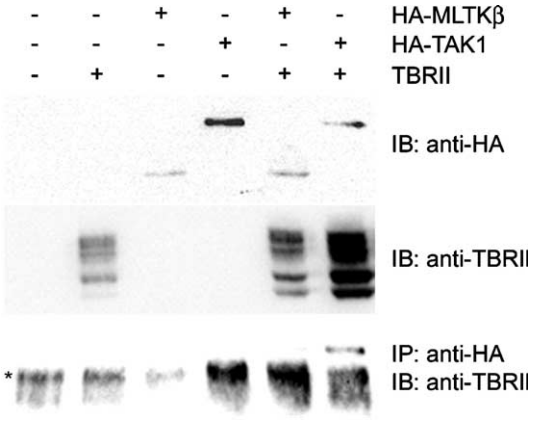

Fig. 4. TBRII and TAK1 interact specifically. Immunoblot analysis of Cos7 cell lysates co-expressing TBRII with either HA-MLTKβ or HA-TAK1 shows that TBRII is not detectable after immunoprecipitation of MLTKβ, but is detectable after immunoprecipitation of TAK1. (*)=Non-specific IgG band.

Red-conjugated secondary antibody, as before. Slides were mounted in Vectashield with DAPI (Vector laboratories). Images were acquired using a Zeiss Axioplan II microscope and Axiovision software.

2.4. Preparation of cell extracts and GST fusion proteins

To prepare protein extracts for immunoprecipitation, cells were harvested in ice-cold NTT lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 8, 1% Triton X-100, 10 mM NaF, +protease inhibitor cocktail [bestatin, leupeptin, pepstatin A, aprotinin, E64; Sigma]) and homogenized through a 25 Gauge syringe needle. Cell debris was removed by microcentrifugation and supernatant was used for immunoprecipitation experiments. GST, GST–Endoglin and GST–TBRII fusion proteins were purified from BL21 *Escherichia coli* extracts using Glutathione Sepharose 4B resin followed by elution with 10 mM reduced glutathione, 50 mM Tris pH 8.

2.5. Immunoblotting and immunoprecipitation

For immunoblot analysis, protein was transferred to nitrocellulose membrane, blocked with PBS/0.3% Tween-20/3% skimmed milk before incubation with primary antibody. Washed membranes were incubated with HRP-conjugated secondary antibody and proteins visualised using ECL. For immunoprecipitation, cell lysates were incubated for 2 to 12 h at 4 °C with the indicated antibody. IgG was precipitated by incubation with sepharose-conjugated protein A (Sigma) for 1 to 2 h at 4 °C followed by microcentrifugation. The immunoprecipitate was washed in high salt NTT buffer (400 mM NaCl, 50 mM Tris–HCl pH 8.0, 1% Triton X-100, 10 mM NaF, +protease inhibitor cocktail) and analyzed by SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting.

2.6. In vitro binding assay

[^{35}S]-methionine labelled TAK1 protein was prepared using a TnT T7 Quick coupled transcription/translation system (Promega) mixed with purified GST and GST fusion proteins, and then precipitated with Glutathione Sepharose 4B resin. GST pull down proteins were washed three times in NTT buffer, separated by SDS-PAGE and radioactivity was detected with a BAS 2000 image analyzer (Fuji).

3. Results

3.1. TAK1 protein interacts specifically with the type II TGFβ receptor (TBRII)

The role of TAK1 in cardiac hypertrophy and its extremely rapid activation by TGFβ1 [14] led us to hypothesise that there may be a direct interaction between TAK1 and a ligand-binding TGFβ receptor. In order to test this hypothesis we performed immunoprecipitation (IP) experiments on 293T cells that co-expressed TAK1 with one of the type I TGFβ receptors (ALK5 or ALK1) or the type II TGFβ receptor (TBRII). Ectopically expressed FLAG-tagged TAK1 protein was immunoprecipitated using monoclonal anti-FLAG antibody. The type I receptors (ALK5 and ALK1) and type II receptor (TBRII) all carried Haemaglutinin (HA) epitope tags at their N-terminus and were all detected in the cell lysates (Fig. 1, 2nd panel). However, immunoblot analysis of the TAK1 immunoprecipitate detected only the TBRII protein and not the ALK5

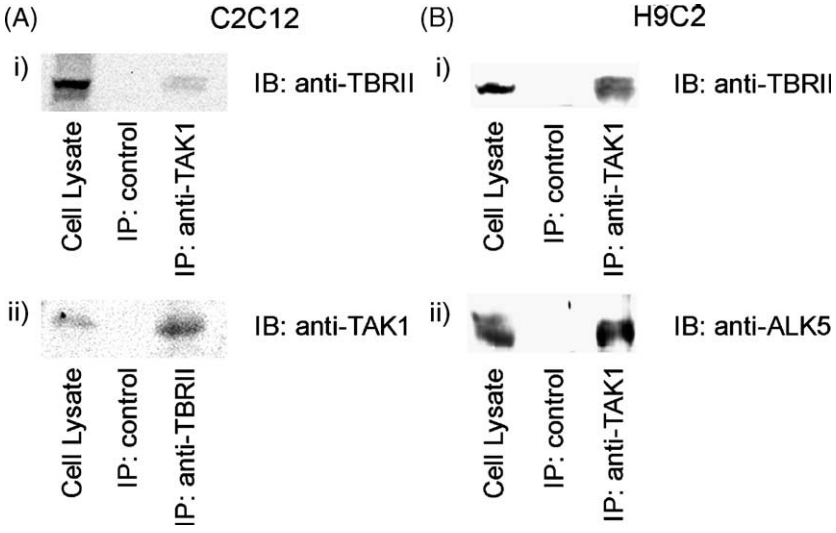

Fig. 5. Endogenous TBRII and TAK1 co-immunoprecipitate. (A) Immunoblot analysis of C2C12 cell lysates detects (i) endogenous TBRII after immunoprecipitation of endogenous TAK1 and (ii) endogenous TAK1 after immunoprecipitation of endogenous TBRII. (B) (i) Immunoblot analysis of H9C2 cardiomyocyte cell lysates with anti-TBRII antibody detects endogenous TBRII after immunoprecipitation of endogenous TAK1. (ii) Further immunoblot analysis of TAK1 precipitates from H9C2 cells with anti-ALK5 antibody also detects endogenous ALK5.

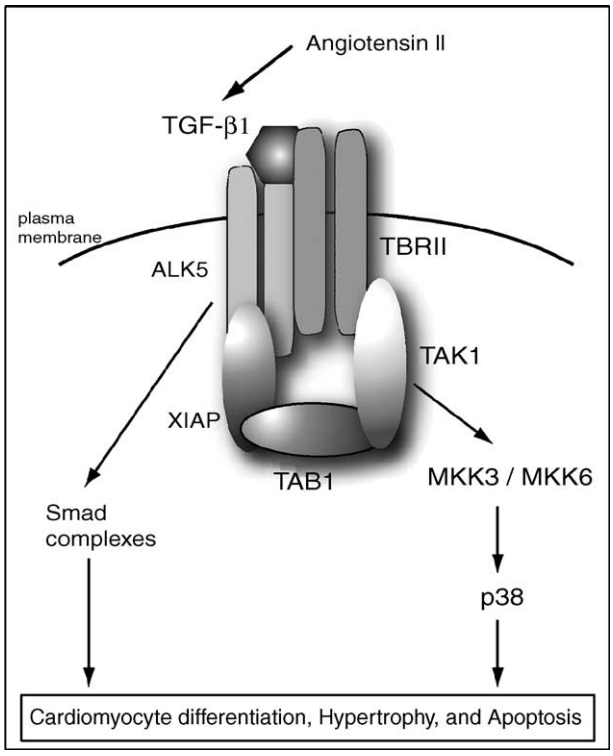

Fig. 6. Model of integrated Smad-dependent and TAK1-dependent TGFβ signalling. TGFβ1 is induced by angiotensin II and binds to the type II TGFβ receptor (TBRII) on the cell membrane. TBRII phosphorylates and activates ALK5 (the type I receptor), which can then propagate the signal via Smad proteins to the nucleus where Smad-dependent TGFβ responsive genes are regulated. ALK5 also binds to the X-linked inhibitor of apoptosis (XIAP), which associates with TAB1 the major binding partner for TAK1 [32] and therefore indirectly links TAK1 to the type I receptor. TAK1 also associates directly with the cytoplasmic domain of TBRII forming a TGFβ-receptor protein complex at the cell membrane. Upon exposure to TGFβ, the TBRII/TAK1 interaction would allow rapid activation of TAK1 via the TGFβ receptor complex. Active TAK1 phosphorylates MKK3/6, which in turn activates p38 [20]. Smad-dependent and TAK-dependent signals may act separately or in conjunction to affect a variety of cell functions. TAK1 signalling appears to be particularly important in regulating cardiomyocyte hypertrophy.

or ALK1 type I receptor proteins (Fig. 1, 3rd panel). Conversely, when all three TGFβ receptors were immunoprecipitated with anti-HA antibody, TAK1 protein could only be detected in the TBRII immunoprecipitate and not in the ALK5 or ALK1 immunoprecipitate (Fig. 1, bottom panel). These results demonstrate that TAK1 interacts with TBRII but not with type I TGFβ receptors. Immunofluorescence microscopy of Cos7 cells co-transfected with both TAK1 and TBRII plasmids showed that cells clearly expressed both TAK1 and TBRII proteins and that there was a significant amount of co-localisation both in the cytoplasm and at areas of the cell membrane (Fig. 2A).

3.2. TAK1 protein can interact directly with TBRII

In order to determine that the TAK1 interaction with TBRII was not due to the presence of any specific intermediate binding proteins, an in vitro binding assay was performed. ^{35}S-labelled TAK1 protein was generated by in vitro transcription and translation and then mixed directly with GST fusion proteins of the cytoplasmic domains of TBRII and the type III TGFβ receptor, Endoglin. GST fusion proteins were precipitated with Glutathione Sepharose resin and precipitates were analyzed for the presence of ^{35}S-TAK1. Radiolabelled TAK1 protein was clearly detectable after precipitation of GST–TBRII protein but not in precipitates of GST–Endoglin or GST alone (Fig. 2B). The in vitro binding assay strongly suggests that TAK1 is capable of directly interacting with the cytoplasmic domain of TBRII and that the co-immunoprecipitation results were not due to the action of an intermediary binding or scaffold protein.

3.3. Three isoforms of TAK1 can interact with TBRII

There are four isoforms of human TAK1 (TAK1A, B, C and D) and all are very similar to each other but vary significantly at their C-Terminal ends [26]. TAK1A is the most abundant across all tissue types and the one we have used in all other experiments. TAK1A, B and C are ubiquitously expressed whereas TAK1D has a more restricted pattern of expression. All four isoforms are expressed in cardiac tissue with TAK1A and B expressed at the highest levels [26]. In order to further characterise the TAK1/TBRII interaction we selected three TAK1 isoforms—A and B (due to their abundance in heart) and D (which differed most at the C terminus—Fig. 3B) and co-expressed them with TBRII in Cos7 cells. All three isoforms carried N-terminal Xpress epitope tags (Promega) and were immunoprecipitated from whole cell extracts with an anti-Xpress antibody (Fig. 3A). Immunoblot analysis with anti-TBRII antibody detected TBRII protein equally in all three immunoprecipitates (Fig. 3A—bottom panel). TBRII therefore interacted with all three TAK1 isoforms tested.

3.4. TBRII interacts specifically with TAK1

To confirm that the TAK1/TBRII interaction is specific to TAK1, TBRII protein was co-expressed in Cos7 cells with either TAK1 or MLK-like triple kinase β (MLTKβ—[27]), a closely related MAPKKK with 45% homology to TAK1. Both TAK1 and MLTKβ proteins were immunoprecipitated from whole cell lysates. Immunoblot analysis with anti-TBRII antibody detected TBRII protein in the TAK1 precipitate but not in the MLTKβ precipitate (Fig. 4). The results confirm that TBRII specifically interacts with TAK1 and not with a related kinase.

3.5. TAK1 and TBRII proteins interact endogenously

Endogenous interactions between TAK1 and TBRII were investigated in both mouse myoblast (C2C12) and rat

cardiomyocyte (H9C2) cell lines. C2C12 cell lysates were immunoprecipitated with anti-TAK1 monoclonal antibody in order to pull down endogenous TAK1 protein. Immunoblot analysis with anti-TBRII antibody clearly detected endogenous TBRII protein in the TAK1 precipitate (Fig. 5A, i). A reciprocal immunoprecipitation with anti-TBRII antibody on C2C12 cell lysate also precipitated endogenous TAK1 protein as detected by immunoblot analysis (Fig. 5A, ii). These experiments confirmed an interaction between TAK1 and TBRII proteins at endogenous levels. Similar experiments were also performed on H9C2 cardiomyocyte cells. Immunoblot analysis with anti-TBRII antibody again clearly detected endogenous TBRII protein in the TAK1 precipitate (Fig. 5B, i). The results confirmed that TAK1 and TBRII proteins interact at endogenous protein levels in cardiomyocyte cells. We also tested whether endogenous TAK1 protein interacted with ALK5 indirectly, perhaps through the known association of ALK5 with TBRII. This was tested in the cardiomyocyte cells by immunoblot analysis of the endogenous TAK1 precipitate with anti-ALK5 antibody and clearly shows that endogenous ALK5 is indeed present in association with TAK1 (Fig. 5B, ii). As TAK1 did not interact directly with ALK5 when both were over-expressed (Fig. 1) it is likely to be an indirect interaction, as indicated in Fig. 6.

4. Discussion

Angiotensin II has been well characterised as a promoter of cardiac hypertrophy [28–30] and more recently it has been shown to trigger the hypertrophic response through TGFβ1 signalling [5,6]. Of the downstream proteins known to be activated by TGFβ signalling, in vivo data suggests that TAK1 is critically important in the cardiac hypertrophic response [14] and TAK1 inhibitors can reduce fibrosis and inflammation [15,16]. However, the mechanism by which (intracellular) TAK1 is rapidly activated in cardiomyocytes by (extracellular) TGFβ ligand remains unresolved. Here we report an important step forward, as we have discovered a novel interaction between TAK1 and the cytoplasmic domain of TBRII that potentially explains the swift response of TAK1 to TGFβ. We used reciprocal co-immunoprecipitations (co-IPs) and in vitro GST pull down assays to show that TBRII binds to TAK1. Consistent with this interaction, immunofluorescence microscopy showed that transiently over-expressed TAK1 and TBRII proteins co-localise in transfected cells. Unfortunately, the detection limits of fluorescent immunocytochemistry in our hands meant that we were unable to ascertain co-localisation of endogenous proteins. The specificity of the interaction was confirmed in that there was no detectable interaction between TBRII and MLTKβ, a kinase closely related to TAK1. Importantly, the interaction was also observed between endogenous TAK1 and TBRII proteins in both mouse muscle myoblast and rat cardiomyocyte cell types. Further detailed characterisation of the interacting sites are currently in progress, but the fact that three TAK1 isoforms (TAK1A, B and D) all interact with TBRII despite significant variation at the C-terminus suggests that this interaction is not mediated at the C-terminal region of the TAK1 protein.

An indirect association between TAK1 and the TGFβ receptors has previously been proposed as the X-linked inhibitor of apoptosis (XIAP) has been reported to interact with TAB1 [31] and to promote association of TAB1 to the type I receptor, ALK5 [32]. The work described here also indicates that TAK1 protein associates indirectly with ALK5, but that this may occur through direct association with TBRII. Taken together, this evidence suggests that a molecular complex is formed at the cell membrane composed of the TGFβ receptors and associated proteins including Smads, XIAP and TAK1 (as described in Fig. 6). We hypothesise that when the complex is exposed to TGFβ, TAK1 becomes activated through its association with the TGFβ receptor complex and phosphorylates downstream MAPKKs. As described above, there is a wealth of evidence pointing to the importance of TGFβ and TAK1 in cardiac hypertrophy so it is likely that these signalling events directly contribute to the hypertrophic response. Furthermore, the direct interaction of TAK1 with TBRII potentially explains the rapid activation of TAK1 (within 1 min) when extracellular TGFβ binds TBRII at the cardiomyocyte cell surface [14]. However, further work is required to determine whether this occurs via phosphorylation of TAK1 by the TGFβ receptor complex. There is considerable evidence for synergy between the TAK-dependent and Smad-dependent TGFβ signalling pathways [32–35]. TAK1 has been reported to interact with Smad 7 to inhibit TGFβ signalling by a negative feedback mechanism [36,37]. More recently, TAK1 has been shown to interact with Smads 1, 2, 3, 4, 5 and 6 [38] and to inhibit BMP signalling. These studies illustrate the close integration of the Smad-dependent and TAK-dependent TGFβ signalling pathways but the relative contributions of TAK-dependent and Smad-dependent pathways to cardiac hypertrophy are still to be determined [39]. Nonetheless, the TAK1/TBRII interaction described here potentially explains how two divergent, but linked, TGFβ responsive pathways can respond to the same extracellular TGFβ signal (Fig. 6).

Our results show a specific interaction between the TGFβ receptor II and TAK1, which is likely to play an important role in regulating TAK1 activity in cardiomyocytes. Further work is in progress to characterise this interaction and investigate the molecular nature of the regulation of TAK1 activity by TGFβ1 in cardiomyocytes. However, even at this early stage, this interaction suggests how TAK1 can be rapidly activated by TGFβ1 and may be a critical molecular mechanism in the initiation of cardiac hypertrophy.

Acknowledgements

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