

**SARA is a Novel Anchoring Protein Essential
for TGF- β Signal Transduction**

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science

Graduate Department of Molecular and Medical Genetics
University of Toronto

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That our affections kill us not, nor dye.

Donne

SARA is a Novel Anchoring Protein Essential for TGF- β Signal Transduction

**Master of Science, 1998
Theodore Andrew Chiang**

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Abstract

In this study, I identify a novel human gene, which is the homolog of a *Xenopus* gene. I present the molecular cloning and characterization of the protein product of this gene, which is, called **SARA** (**S**mad **A**nchor for **R**eceptor **A**ctivation). SARA is a novel component of the TGF- β signal transduction pathway. It interacts directly with Smad2, a cytoplasmic mediator of TGF- β signalling and functions to recruit Smad2 to specific subcellular sites of action. I also demonstrate that TGF- β receptors localize to these regions in the cell and show a physical association of SARA with receptor complexes. I characterize the SARA domains required for proper subcellular localization and Smad2 interaction, and demonstrate that a mutant version of this protein blocks Smad2 signalling. Based on these characterizations, I propose that SARA functions to recruit Smad2 to the TGF- β receptor by controlling its subcellular localization.

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Chapter 1

Introduction

The TGF- β Signal Transduction Network

Introduction The TGF- β Signal Transduction Network

The TGF- β (Transforming Growth Factor- β) family of signalling polypeptides regulates and exerts a wide range of biological effects. These processes include cell growth, differentiation, apoptosis and development (reviewed in Hoodless and Wrana, 1997 and Massagué, 1998). For example, members of this family control early embryogenesis (reviewed in Hogan, 1996) and contribute to the development of various forms of human cancers (reviewed in Massagué and Weis-Garcia, 1996 and Markowitz and Roberts, 1996). Intensive studies over the last few years have elucidated intracellular molecular mechanisms and components of the signal transduction network that mediate the cellular responses of these growth factors. Thus far, three important repertoires of molecules have been defined in this pathway: (1) cell surface receptor ser/thr kinases, (2) intracellular mediator Smads, and (3) nuclear targets and DNA-binding partners.

Surface Receptors

The biological effect of a ligand depends largely on the responsiveness of the target cell. Response to ligands is mediated on the cell surface through specific types of membrane receptors. Members of the TGF- β superfamily signal through two receptor ser/thr kinases, and they have been designated type I and type II receptors (reviewed in ten Dijke et al., 1996). In the TGF- β signalling pathway, receptors known as T β RII (type II) and T β RI (type I) form an active receptor complex (Wrana et al., 1994). TGF- β receptor activation begins when ligand binds the type II receptor T β RII and causes recruitment of the type I receptor T β RI. This results in the formation of an active heteromeric receptor complex. As a constitutively active kinase, the type II receptor will transphosphorylate the type I receptor in a juxtamembrane region, known as the GS domain. Phosphorylation of T β RI activates the type I receptor kinase and allows it to propagate the signal by targeting downstream substrates. Studies of receptor activation

have led to the discovery of mutations in the GS domain that constitutively activate T β RI, the downstream component in the receptor complex (Wieser et al., 1995). Thus the events that transduce TGF- β signals at the membrane involve the action of two receptors, one that mediates the activation of a second receptor which in turn targets downstream components.

Smad Proteins

A family of proteins known as Smads has been identified as cytoplasmic mediators that propagate signals from the type I receptor (reviewed in Attisano and Wrana, 1998; Kretzschmar and Massagué, 1998 and Padgett et al., 1998). Thus far, eight Smad proteins have been identified in mammals, and they can be divided according to their function into three classes: (1) receptor-regulated Smads, (R-Smads) which include Smads 1, 2, 3, 5, 8; (2) common Smads, Smad4; and (3) antagonistic Smads, Smad6 and 7. R-Smads are direct signal transducers and substrates of activated membrane receptor complexes (Macías-Silva et al., 1996 and Kretzschmar et al., 1997). Characteristic of these Smads is the presence of a highly conserved carboxy-terminal amino acid sequence, the SSXS motif. The last two serines of this motif are the phosphorylation sites of the type I receptor kinase (Abdollah et al., 1997; Souchelnytskyi et al., 1997 and Kretzschmar et al., 1997). Phosphorylation of Smad2 and Smad3 are important for mediating TGF- β and activin signalling (Macías-Silva et al., 1996; Liu et al., 1997 and Nakao et al., 1997), whereas Smad1, 5 and 8 transduce signals from the type I receptors for Bone Morphogenetic Proteins (BMPs; Hoodless et al., 1996; Macías-Silva et al., 1998; Nishimura et al., 1998 and Chen et al., 1997). Activation of R-Smads induces their nuclear translocation, and mutations that prevent receptor-dependent phosphorylation will also block entrance to the nucleus (Macías-Silva et al., 1996). In addition, phosphorylation of Smads results in the formation of heteromeric complexes with Smad4, the only common mediator Smad thus far identified in mammals (Lagna et

al., 1996; Zhang et al., 1996 and 1997; Wu et al., 1997). The structure of Smad4 is similar to R-Smads and the protein normally resides in the cytosol. However, Smad4 does not contain a SSXS motif and is not a substrate of the receptor. Thus, Smad4 interaction with an R-Smad is required for its nuclear translocation (Liu F et al., 1997). The third class of Smads can be designated as anti-Smads, consisting of Smad6 and Smad7. They inhibit the signalling function of receptor-regulated Smads by blocking their access to the type I receptors or by disrupting Smad4 heteromeric complexes (Hayashi et al., 1997; Topper et al., 1997; Nakao et al., 1997; Imamura et al., 1997 and Hata et al., 1998).

Structurally, Smad proteins can be divided into three domains (reviewed in detail by Heldin et al., 1997 and Massagué, 1998). The amino and carboxy terminal regions designated respectively as MH1 and MH2 (MAD Homology 1 and 2) are conserved domains found in all members of the Smad family. The central portion of Smads separating these two domains is a non-conserved region, which is termed the Linker region. Numerous functions have recently been characterized for each Smad domain. The MH1 domain can act to associate with the MH2 domain, potentially functioning in a mutually repressive intramolecular interaction (Hata et al., 1997). In addition, the MH1 domain in some Smads possesses DNA-binding activity and can interact with transcription factors (Kim et al., 1997; see Nuclear Function below). The MH2 domain also carries out multiple functions. In R-Smads, it contains the receptor phosphorylation sites and interacts with the type I receptor kinase (Macías-Silva et al., 1996; Kretzschmar et al., 1997). The MH2 domain has the ability on its own to activate and regulate expression patterns in the *Xenopus* embryo, (Baker and Harland, 1996 and Meersseman et al., 1997) and can activate transcription when fused to heterologous DNA-binding domains (Liu et al., 1996). The MH2 domain is also important for activation-induced association with Smad4 (Hata et al., 1997) and with DNA-binding partners in the nucleus (Chen et al., 1997). Finally, in R-Smads the Linker domain contains MAP-kinase

phosphorylation sites (Kretzschmar et al., 1997) and a PY recognition motif for WW domains (Chen et al., 1995) although no interacting partners have been identified or reported.

Nuclear Targets and Function

The translocation of Smad complexes into the nucleus led to the hypothesis that Smad proteins play an important role in gene transcription. These nuclear functions were first suggested in studies demonstrating that the MH2 domain of Smad1 and Smad4 could activate transcription (Liu et al., 1996). Soon afterward FAST-1, the first transcriptional partner for Smads was identified. FAST-1 is a novel *Xenopus* winged-helix transcription factor that interacts specifically with Smad2 (Chen et al., 1996). FAST-1 functions in the regulation of the transcription of the early response gene *Mix.2* by recognizing a DNA regulatory element present in the promoter. Ligand-dependent activation of this promoter occurs when Smad2 binds FAST-1 to form a DNA-binding complex. Thus, Smad2 was shown to transduce signals from the cell surface to the nucleus. The observation that Smad2 accumulates in the nucleus in response to ligand (Baker et al., 1996 and Macías-Silva et al., 1996), taken together with the finding that the formation of a Smad2-Smad4 complex is also dependent on ligand activation (Lagna et al., 1996 and Zhang et al., 1997) led to the demonstration that Smad4 is translocated into the nucleus in a complex with Smad2 and is involved in activation of this transcriptional complex (Liu et al., 1997 and Chen et al., 1997).

Thus, the participation of Smads in transcriptional complexes appeared to involve the formation of a higher order complex consisting of activated Smads and specific DNA binding proteins. However, further elucidation of Smads' role in the nucleus led to the finding that Smads could bind DNA directly, and activate transcription. This was first demonstrated with the MH1 domain of *Drosophila* MAD, which binds directly to a dpp responsive element in the quadrant enhancer of the *vestigial* gene (Kim et al., 1997).

This report also showed that the DNA binding activity is repressed in the presence of the MH2 domain, supporting the notion that Dpp-dependent activation of MAD phosphorylation stimulates nuclear translocation, DNA binding and the subsequent expression of target genes. DNA binding activity for mammalian Smads was also described for Smad3 and Smad4 binding to the TGF- β inducible reporter 3TP-lux (Yingling et al., 1997), to the promoter of human plasminogen activator inhibitor-type 1 gene (Dennler et al., 1998; Hua et al., 1998 and Song et al., 1998), in an artificial sequence-specific palindromic element (Zawel et al., 1998) and in the promoter of the JunB gene, an immediate early gene induced by members of the TGF- β family (Jonk et al., 1998). Thus, there is mounting evidence that Smads fulfill important functions through direct binding to DNA regulatory elements. The recent crystal structure of the MH1 domain bound to DNA has provided a structural basis of the conserved regions, which are important for this interaction (Shi et al., 1998).

The exact role of Smads in gene transcription may rely on the necessity for both the physical interaction of Smads with specific DNA regulatory sequences as well as the presence of a nuclear binding partner. Recent studies describe Smads in a number of other nuclear transcriptional complexes; 1) the repressor Evi-1, which interacts and suppresses the transcriptional activity of Smad3 (Kurokawa et al., 1998); 2) the transcriptional coactivators p300/CBP, which binds Smads in mediating transcriptional activation events (Janknecht et al., 1998; Feng et al., 1998; Topper et al., 1998; Pouponnot et al., 1998); 3) the DNA transcription factors AP-1 composed of c-Jun and c-Fos, which together with Smad3 and Smad4 can bind DNA and activate transcription in response to TGF- β (Zhang et al., 1998); 4) the orphan transcriptional activator MSG1, which lacks DNA-binding activity but functionally interacts with Smad4 (Shioda et al., 1998), and finally, 5) Smads in a TGF- β specific protein-DNA complex in the transcriptional activation of human type VII collagen gene (COL7A1) (Vindevooghel et al., 1998). Some of these reports show additionally a combination of both Smad binding to

DNA and to a transcription factor in order to effect an efficient mechanism of transcriptional regulation. This notion is well described in the recent documentation of two mammalian FAST proteins identified as hFAST-1 and FAST-2 (Zhou et al., 1998 and Labbé et al., 1998). Both reports demonstrate ligand-dependent transcription is mediated by a DNA-binding complex formed by FAST, Smad2, and Smad4. Moreover, formation of DNA binding complexes and efficient activation of transcription requires Smad4 binding to a Smad Binding site that lies adjacent to the FAST site. In addition, Labbé et al., also report that FAST target promoters can be negatively regulated by Smad3 through competitive binding of Smad3 to the Smad4 site. This provides an interesting mechanism whereby a target gene can be positively and negatively regulated.

Thus, it appears the nuclear roles of Smad proteins can be dependent on the formation of a higher order DNA transcriptional complex as well as the binding of Smads to specific DNA Elements, or through both functions. Evidently, the final critical step in ligand-induced gene expression requires the transcriptional regulation of specific target genes, and Smads have established an important role in this mechanism.

A Model for TGF- β Signal Transduction

The TGF- β molecular signalling pathway undergoes a number of critical events from the moment of extracellular stimulation to the eventual transcription of target genes in the nucleus. In sum, the pathway is activated when TGF- β binds two distinct receptors at the cell surface and induces the formation of an activated receptor complex. This allows the intracellular kinase of the type I receptor, T β RI to activate and phosphorylate the downstream target, Smad2. This phosphorylation induces a Smad2-Smad4 heteromeric complex which translocates into the nucleus to direct the transcription of target genes. Activation or repression of responsive genes is regulated through the interaction of Smad complexes with both specific nuclear factors, such as FAST-1 and

direct binding to DNA. A current model for TGF- β signal transduction is summarized in Figure 1.

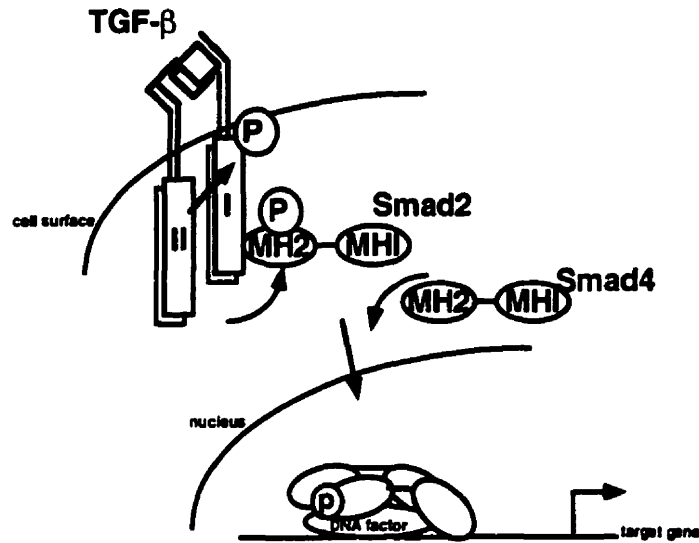


Figure 1. TGF- β Signal Transduction model. An activated receptor complex is formed at the membrane when ligand is bound. Phosphorylation of the T β RI activates the pathway and allows it to phosphorylate the substrate, Smad2. Activated Smad2 forms a heteromeric complex with Smad4 which translocates into the nucleus to activate target genes.

Thesis Rationale and Objective

Although recent advances have been made in the field of TGF- β signal transduction, little is known about the cytosolic nature of Smad2 regulation and the precise mechanism of Smad2 recruitment to the TGF- β receptor. One method to better understand these processes is to identify Smad2-interacting proteins. To this end, a postdoctoral fellow in the lab, Tomoo Tsukazaki isolated a Smad2 binding protein by screening a *Xenopus* expression library constructed in λ phage. In his initial biochemical studies, he identified a region in this clone that is important for Smad2 binding.

These observations provided the groundwork for the initiation of my study which can be divided into two parts. My first objective was to identify the corresponding human gene product of this *Xenopus* clone. To this end, I utilized a degenerate cloning strategy based on the amino acid sequence of the *Xenopus* clone. My second objective

was to characterize the role of this clone with respect to TGF- β and Smad2 signalling.

To achieve this, I investigated the molecular function of this clone in mammalian cells in response to TGF- β signalling.

As a result, I report the identification and characterization of a novel Smad2 interacting protein now called SARA, and help define its role in TGF- β signal transduction.

Chapter 2

Results

(note: The results in this section have been published under the title: **SARA, a FYVE Domain Protein that Recruits Smad2 to the TGF β Receptor**. Tomoo Tsukazaki, Theodore A. Chiang, Anne F. Davison, Liliana Attisano, and Jeffrey L. Wrana. Cell 95(6), 779-791. Dec 11th 1998.)

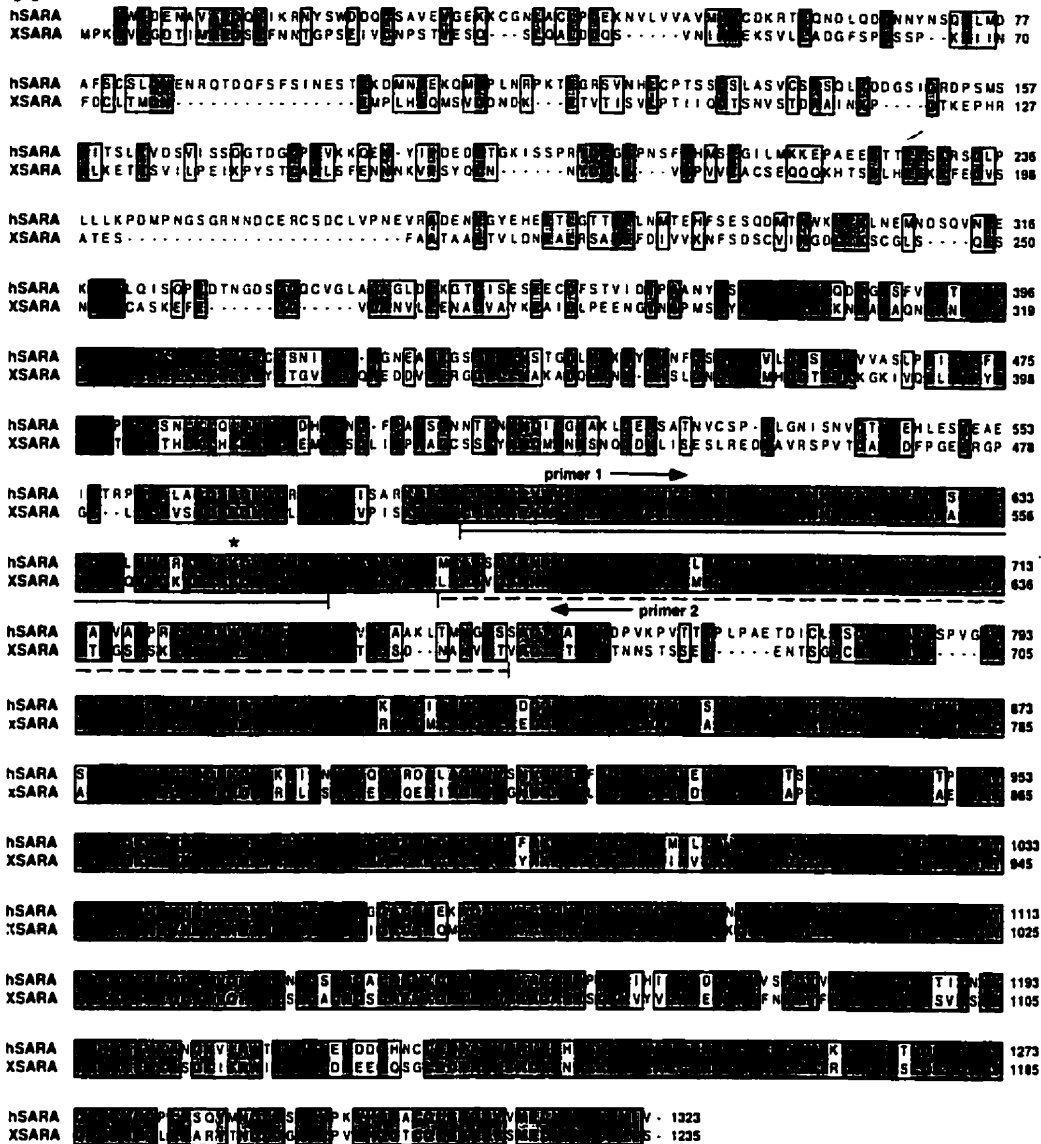
Results

I. The Identification of SARA

Cloning of hSARA

Based on the knowledge that *Xenopus* SARA contained a domain important for Smad2 interaction, it seemed likely that potential human homologs would retain a high degree of amino acid conservation across this region. Thus to identify human SARA, I utilized a two step cloning strategy that involved the use of degenerate PCR, followed by screening a cDNA library. First, I designed degenerate primers that corresponded to this binding region (Figure 2) and utilized RNA from HepG2 cells in a RT-PCR reaction. This yielded in a PCR product of 250 bp which was sequenced and found to be homologous to *Xenopus* SARA . To isolate a full length clone, I designed a probe based on the sequence of this PCR fragment to screen a human fetal brain cDNA library which resulted in the identification of one partial cDNA clone that encoded the 5' half of a hSARA-like cDNA. Searches in the GenBank EST database identified a partial clone that appeared to encode the 3' half of hSARA (EST#183440). Sequence analysis revealed that both partial clones were derived from the same gene product, and had identical sequences across an overlapping region of approximately 1 Kb. The fusion of both clones at the restriction enzyme site *Stu*I produced a full-length clone predicted to be 1323 amino acids long. This clone is now called hSARA for Smad Anchor for Receptor Activation. The comparison of hSARA and xSARA protein sequences revealed a poorly conserved amino terminal segment (35% identity: aa 1-570), and a highly conserved carboxy terminal region (85% identity: aa 571-1323). The overall amino acid identity was calculated at 62%, and the sequence alignment is shown in Figure 2A.

A



B

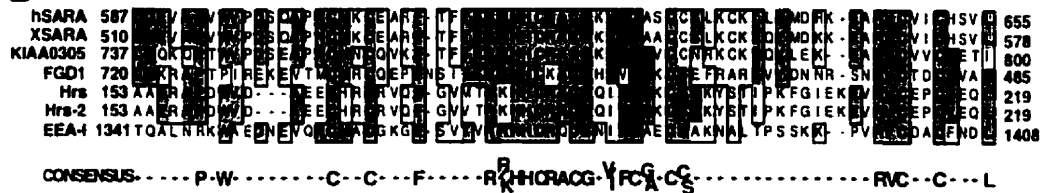


Figure 2. A. The amino acid sequence alignment of both *Xenopus* and Human SARA. The solid line denoted under the sequences represents the region known as the FYVE-finger, and the dotted line represents the Smad Binding Domain, SBD (see text and following Figures). The arrows designated as primer 1 and 2 are the regions where degenerate PCR primers based on the xSARA sequence were designed. The PCR product of degenerate RT-PCR reactions from the human cell line, HepG2 was used as a probe to screen a human cDNA library in order to clone the human homolog, hSARA.

B. The amino acid sequence alignment of the FYVE-fingers from various proteins. xSARA and hSARA contain the conserved residues of the FYVE domain. On the bottom, a consensus sequence of important residues is shown.

Database Analysis of SARA reveals a FYVE-finger

The sequence analysis in GenBank databases revealed that hSARA and xSARA are not part of any known family of proteins. However, these searches identified a domain known as a FYVE-finger located in the center portion of SARA (FYVE: aa 587-655 for hSARA and aa 510-578 for xSARA). The FYVE-finger of hSARA and xSARA is 96% identical, and highly conserved in comparison to FYVE fingers from other proteins that contain this domain (Figure 2B). The FYVE-finger is a **double zinc-finger** domain highlighted by eight conserved cysteine residues and a signature patch of basic amino acids surrounding the third cysteine residue (RRHHCR). This is summarized in the consensus line at the bottom of Figure 2B. The mammalian FYVE-finger proteins EEA1, Hrs, and FGD1 do not appear to be related to each other and outside of the FYVE domain have no similarity to SARA. This suggests that FYVE fingers may possess a common functional role in various structurally distinct proteins, and recent findings indicate that the FYVE-finger of EEA1 and Hrs (GST-FYVE fusion proteins) function as structural motifs sufficient to bind the membrane lipid, PtdIns(3)P with high specificity (Gaulhier et al., 1998; Burd et al., 1998; Patki et al., 1998 and Simonsen et al., 1998). This strongly implicates that the FYVE-finger of SARA may have a similar function and defines a potential role for SARA at the membrane.

II. The Characterization of Smad-SARA Interactions

SARA binds Smad2 and Smad3

The cloning and isolation of the full-length cDNA of *human* SARA allowed me to first test the interaction properties of SARA with Smads. To achieve this, I used an in vitro transcription - translation system to produce [³⁵S]-methionine labelled SARA, and isolated various Smad proteins expressed in bacteria as GST-fusion constructs. The mixture of translated metabolically labelled SARA was incubated with GST-Smad

proteins immobilized on glutathione sepharose beads. The beads were washed extensively and products bound to the Smad proteins were resolved by SDS-PAGE and visualized by fluorography. The predicted full-length product of SARA (and a truncated product) bound specifically to Smad2 and Smad3, while no association with Smad1 and Smad4 was detected. Furthermore, closer examination of this interaction using individual domains of Smad2 fused to GST revealed that SARA bound only to the MH2 domain with no detectable association with the MH1 or Linker domains (Figure 3).

To further characterize this interaction *in vivo*, transient transfection experiments were carried out in COS cells, and they demonstrated similar results. Thus, analysis of cells expressing Flag-tagged SARA and individual Myc-tagged Smads (Smads 1,2,3,4,6,7) by immunoprecipitation followed by western blotting revealed that SARA interacted only with Smad2 and Smad3 (These experiments were performed by Tomoo Tsukazaki and are not shown). The interaction of SARA with both Smad2 and Smad3 is consistent with the high degree of similarity between their two MH2 domains (97% identity), and the role of both proteins as intracellular mediators of TGF- β signalling.

SARA contains a Smad Binding Domain, SBD

The aforementioned data revealed that a region in SARA is important for Smad2 binding. To map this domain, a series of deletion constructs was generated using a combination of restriction enzyme digestion, PCR and ligations as summarized in Figure 4. Each construct was co-expressed with Smad2 in COS cells and assayed for interaction as described above. Based on these results, a region of 85 amino acids (aa 665-750) was identified to be necessary and sufficient for interaction with Smad2, and is now termed the Smad Binding Domain (SBD).

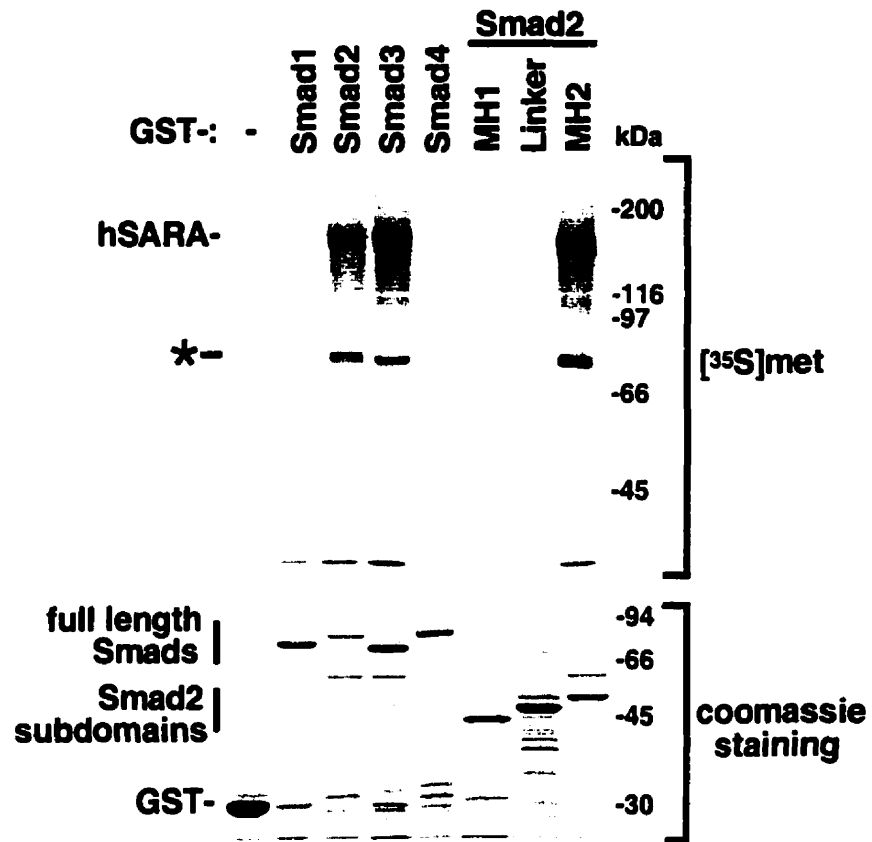


Figure 3. The interaction of SARA with Smads or domains of Smad2. SARA was produced using an in vitro transcription / translation system in the presence of $[^{35}\text{S}]$ -met. The protein product was incubated with bacterially expressed GST-fusion proteins that were immobilized on glutathione beads. Radiolabelled SARA binds specifically to Smad2, Smad3 and the MH2 domain of Smad2. The asterisk indicates a truncated product of SARA present in the translation mixture. The bottom panel shows the relative amount of GST-fusion protein used.

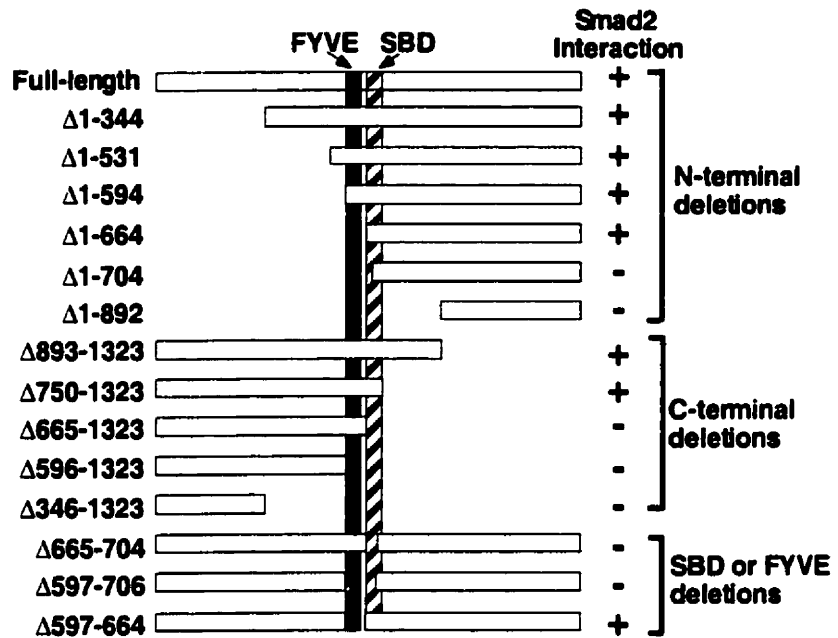


Figure 4. The mapping of the Smad Binding Domain, (SBD) of SARA.

A series of deletion mutants were generated and tested in COS cells for interaction with Smad2 by immunoprecipitation of SARA mutants followed by western blotting for Smad2. These results define residues 665-750 as essential for Smad2 binding.

Smad2 Phosphorylation causes Dissociation from SARA

The in vitro and in vivo experiments suggest that SARA interacts with unphosphorylated Smad2, and thus leads to the question of whether Smad2 phosphorylation might regulate this interaction.

Tomoo Tsukazaki first investigated this in mammalian cells. Briefly, SARA-Smad2 complexes were analyzed in the presence of the wild type and constitutively active type I receptor, T β RI. While complexes were readily detectable in the absence of signalling, activation of Smad2 disrupted interaction with SARA suggesting that receptor phosphorylation of Smad2 causes the dissociation of the complex (data not shown). Furthermore, these effects were examined in the presence of Smad4 which revealed that in the presence of constitutive receptor activation, the dissociation of SARA-Smad2 complexes occurred concomitantly with the formation of Smad2-Smad4 heteromeric complexes (Figure 5). In effect this establishes the order of signalling events, implicating that SARA-Smad2 complexes exist upstream of the receptor activation, and dissociation of this complex is dependent upon receptor phosphorylation which then results in Smad2-Smad4 complexes.

To further characterize how phosphorylation might disrupt interaction with SARA, I tested whether phosphorylation of Smad2 was able to interact with bacterially produced SBD. To investigate this, I utilized an in vitro GST-pulldown assay to assess the ability of GST-SBD to bind Smad2 expressed in COS cells (Figure 6). Briefly, the GST-SBD fusion construct was purified from bacteria and immobilized on glutathione beads, and then incubated with the lysates of COS cells coexpressing Smad2 together with either wild type or constitutively active T β RI. The beads were washed and subjected to SDS-PAGE and western blotting to determine the level of Smad2-SBD interaction. In cells where Smad2 is expressed alone, there is strong interaction with GST-SBD (Figure 6, Lane2). This association is comparable in the presence of wild type T β RI (Lane3), however is significantly reduced in cells expressing constitutively active T β RI (Lane4),

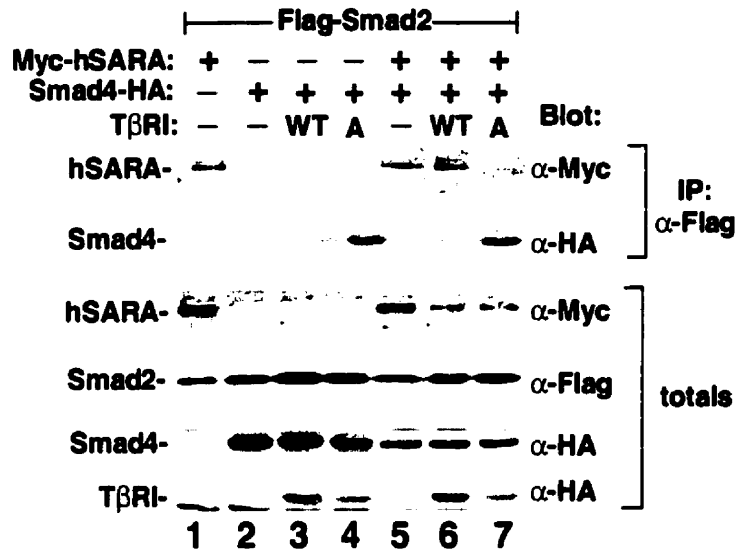


Figure 5. Receptor kinase phosphorylation of Smad2 induces the dissociation of SARA-Smad2 complexes and the formation of Smad2-Smad4 complexes. COS cells were transfected with the indicated combinations of Smad2, Smad4 and SARA and assayed for proteins that bind Smad2 in the absence and presence of receptor activation. Cell lysates were subjected to Flag immunoprecipitation followed by western blotting for coprecipitating proteins. These results show that Smad2 interacts with SARA in the absence of receptor activation, but only with Smad4 in the presence of activation, suggesting its dissociation from SARA. (This experiment was performed by Dr. Tomoo Tsukazaki.)

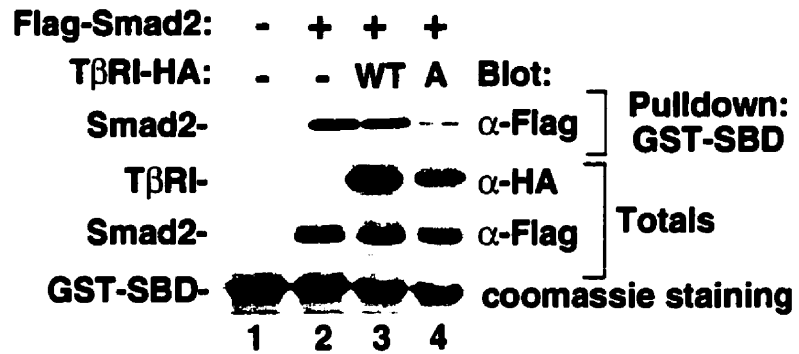


Figure 6. The Smad Binding Domain of SARA interacts preferentially with unphosphorylated Smad2. GST-SBD fusion protein prepared from bacteria and immobilized on glutathione beads was incubated with COS lysates prepared from cells expressing either unphosphorylated, or receptor activated and phosphorylated Smad2. Smad proteins pulldown by the GST-SBD were separated by SDS-PAGE and visualized by western blotting. This result shows that the SBD binds unphosphorylated Smad2 more efficiently than activated Smad2. Coomassie staining shows the relative amount of GST-SBD used.

suggesting that phosphorylated Smad2 interacts poorly with SBD. Thus, the reduced binding capacity of SARA-SBD with receptor phosphorylated Smad2 reveals that SARA interacts preferentially with unphosphorylated Smad2 and supports the above suggestion that receptor-dependent phosphorylation of Smad2 causes it to dissociate from SARA.

III. SARA functions to recruit Smad2 to TGF- β receptors

Subcellular Localization of SARA/Smad2 complexes

Previous reports have demonstrated that the subcellular localization of Smad2 in transfected cells is predominantly cytosolic in unstimulated cells, and activation of TGF- β or activin signalling will induce Smad2 to accumulate in the nucleus. The translocation of Smad2 into the nucleus upon signal initiation, taken together with the above data that SARA-Smad2 complexes exist upstream of receptor activation suggest that SARA may function as a cytosolic anchor for Smad2. Therefore, I explored the nature of the subcellular localization of SARA-Smad2 complexes by immunofluorescent confocal microscopy (Figure 7). Mv1Lu cells transfected with SARA and/or Smad2 were fixed, permeabilized and incubated with appropriate primary antibodies, followed by secondary antibodies conjugated to FITC or Texas Red. SARA was found to be distributed throughout the cell as distinct punctate speckles (Figure 7A) while overexpressed Smad2 had diffuse localization and was evenly distributed throughout the cytoplasm and nucleus (Figure 7B). In cells overexpressing Smad2 along with activated T β RI, there was an accumulation of Smad2 in the nucleus (Figure 7C). Interestingly, in cells coexpressing SARA and Smad2, there was a dramatic shift in Smad2 localization (Figure 7D) to the SARA-containing punctate subcellular regions. Overlay of the red and green filters show that SARA and Smad2 were completely colocalized in the cytoplasm (see yellow in Figure 7Diii). During TGF- β signalling, the colocalization of SARA and Smad2 was

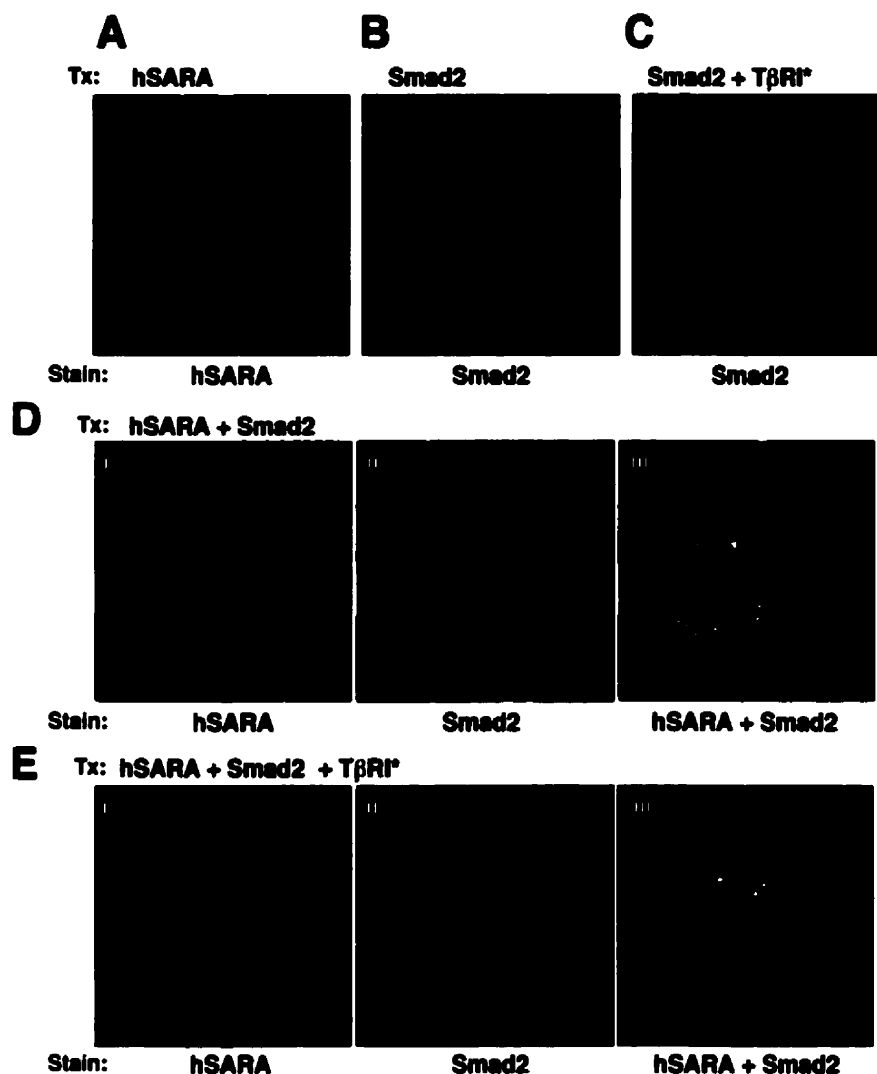


Figure 7. The intracellular localization of Smad2 is controlled by SARA. Mv1Lu cells were transfected with the indicated cDNA and their subcellular localization was assessed by immunofluorescence and confocal microscopy. Cells display a punctate staining pattern for SARA(A), an evenly diffused staining for Smad2 (B), and a predominant nuclear staining for Smad2 in the presence of receptor activation (C). The redistribution of Smad2 localization into SARA punctate domains is observed in cells coexpressing SARA and Smad2 (D). Receptor activation in these cells show a noticeable reduction of Smad2 in punctate domains and an accumulation in the nucleus (E). Colocalization of SARA and Smad2 is indicated as yellow in overlay images (Diii and Eiii). (Tx: transfection, Stain: α flag-Smad2, α myc-SARA, TβRI*: constitutively active TβRI)

decreased with a concomitant increase in nuclear staining of Smad2 (Figure 7E).

Therefore, the intracellular localization of SARA-Smad2 complexes suggest that SARA might function in TGF- β signalling to anchor and recruit Smad2 into punctate domains of the cell.

The FYVE finger of SARA is Important for Localization

The FYVE finger has been reported in EEA1 to be responsible for subcellular targeting (Stenmark et al., 1996). To test whether the FYVE domain of SARA mediates the punctate localization, a number of SARA mutants were expressed in Mv1Lu and protein localization examined by immunofluorescent confocal microscopy (Figure 8). Deletion constructs lacking either the amino or carboxy terminus but not the FYVE domain displayed a localization pattern similar to wild type SARA (Figure 8 ii or iv). However, amino or carboxy terminal deletions that included the FYVE domain were not localized to punctate domains but rather existed diffusely in the cytosol (Figure 8 iii and v). Similarly, deletion of a region within SARA that removed the FYVE-finger (Δ 597-664) also mislocalized SARA (Figure 8 vi). This demonstrates that the FYVE finger functions to localize SARA to subcellular punctate regions in the cell.

Subcellular Localization of SARA and TGF- β Receptors

Observations that SARA interacts with Smad2 and controls its subcellular localization in the absence of TGF- β signalling implied that SARA may function to anchor Smad2 in specific subcellular regions for activation by the TGF- β receptor. Therefore, to investigate whether the SARA domains might contain TGF- β receptors, immunofluorescence and confocal analysis of cells coexpressing SARA and T β RII was performed (Figure 9). The distribution pattern of T β RII in Mv1Lu cells was similar to previous reports of TGF- β receptor localization (Figure 9B, and Henis et al., 1994) and very similar to SARA's distinct punctate speckles (Figure 9). Interestingly, in TGF- β

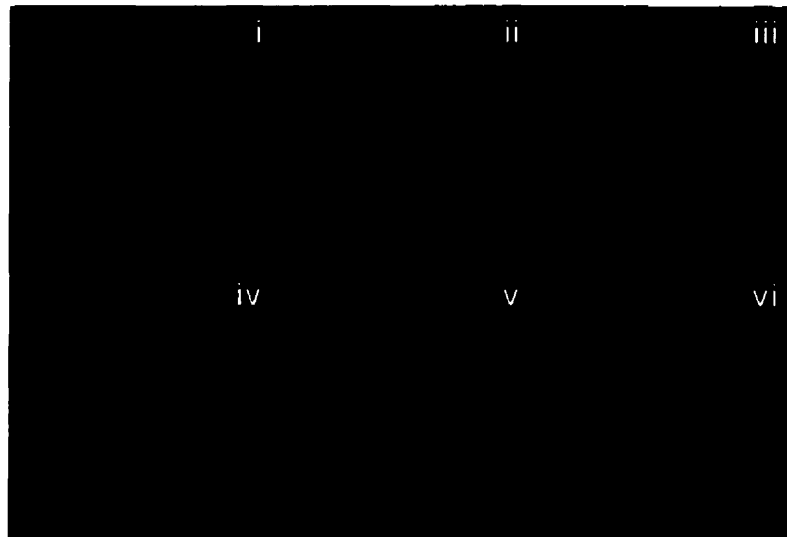
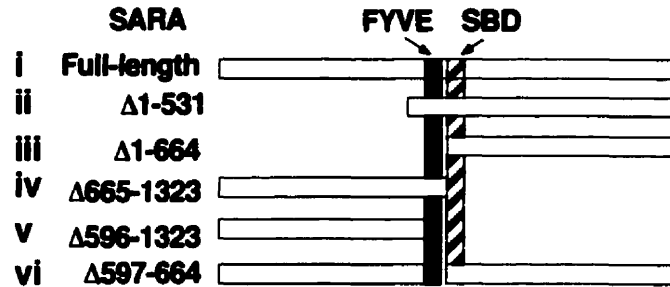


Figure 8. The FYVE-finger of SARA is important for localization. Mv1Lu cells were transfected with the wild type or mutant forms of SARA shown schematically in the top panel. The subcellular localization of these constructs were analyzed by immunofluorescence and confocal microscopy (bottom panels). Amino and carboxy mutants lacking regions including the FYVE-finger are mislocalized (iii and v), whereas mutants retaining the domain localize similarly to wild type SARA (ii and iv). The mutant lacking only the FYVE-finger is mislocalized as well (vi). This result demonstrates the necessity of the FYVE-finger in targeting SARA to subcellular punctate compartments. (Stain: α flag-SARA)

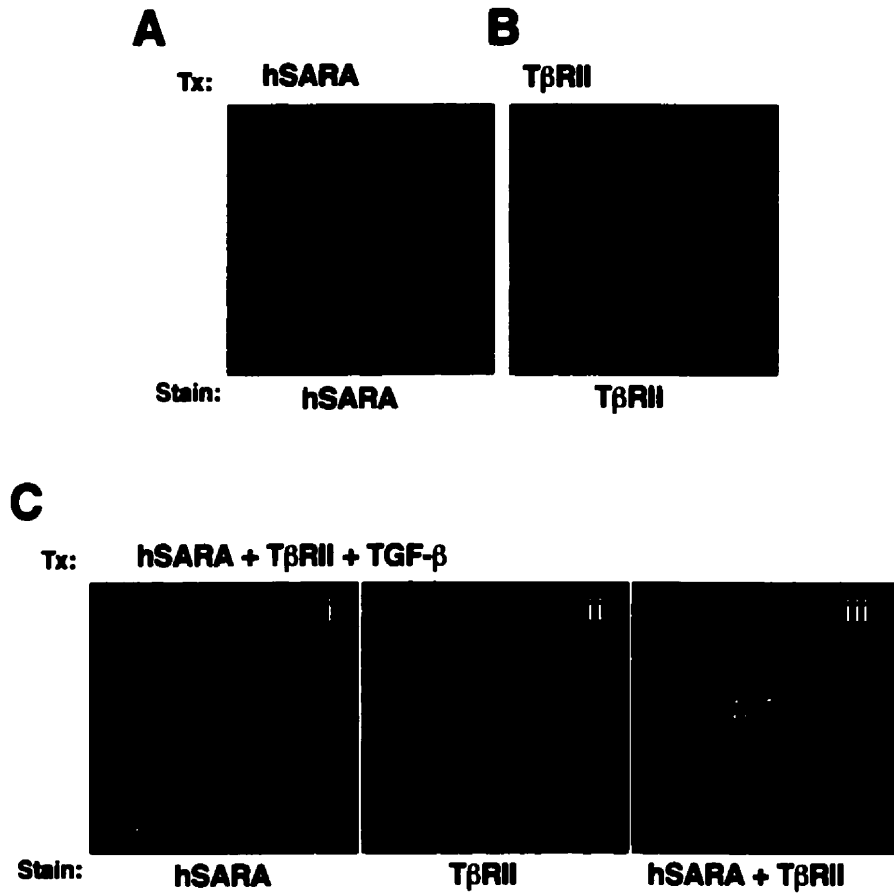


Figure 9. The subcellular colocalization of SARA with TGF- β receptors. Mv1Lu cells were transiently transfected with SARA (A), the type II receptor T β RII (B), or both (C), and their subcellular localization were analyzed by immunofluorescence and confocal microscopy. Cells expressing T β RII displayed a punctate distribution pattern similar to SARA (compare A and B). In cells where SARA and receptor are coexpressed and stimulated with TGF- β , colocalization can clearly be observed as yellow spots (iii) in overlay images of SARA (i) and receptor (ii), however regions with no colocalization are also present. This result demonstrates that SARA and the TGF- β receptor exist in the same subcellular domain. (Stain: α myc-SARA, α HA-T β RII)

stimulated cells expressing SARA and T β RII, I observed extensive colocalization of the two proteins (yellow staining in Figure 9C) although some regions were found where no colocalization was present. These results support the notion that SARA functions to anchor Smad2 in specialized subcellular sites where receptor activation may occur.

Association of SARA with the TGF- β receptor

Although SARA is not a substrate of T β RI, it is a component of the pathway upstream of Smad2 phosphorylation. The colocalization studies of SARA and TGF- β receptors suggest the possibility that SARA may physically associate with TGF- β receptors. Previous findings have shown that Smad2 interaction with receptor complexes can be detected between Smad2 and receptor complexes containing kinase deficient T β RI (K-R), or between the mutant, Smad2 (2S-A), and wild type receptor complexes (Macias-Silva et al., 1996). To visualize these interactions, COS cells expressing Smad2 and TGF- β receptors (T β RII and T β RI) were incubated with [125 I]-TGF- β to induce the formation of a radioactive receptor complex. Lysis of these cells followed by Smad2 immunoprecipitation resulted in the coprecipitating of radioactive receptor complexes which were then detected by SDS-PAGE and autoradiography (Macias-Silva et al., 1996).

I followed a similar strategy to determine whether SARA associates with TGF- β receptor complexes. In these experiments, the association of SARA with receptor complexes was detectable in both the absence and presence of Smad2 (Figure 10). In the absence of Smad2, this association was slightly enhanced using a kinase deficient type I receptor (T β RI-(KR)), in comparison to wild type T β RI (lanes 2 and 3). Interestingly in cells where Smad2 was expressed, there was no significant enhancement of SARA interaction with wild type receptors (lane 5), however there was an appreciable enhancement in the presence of T β RI-(KR) (lane 4). This observation is consistent with previous findings that association of Smad2 with wild type T β RI is transient in nature,

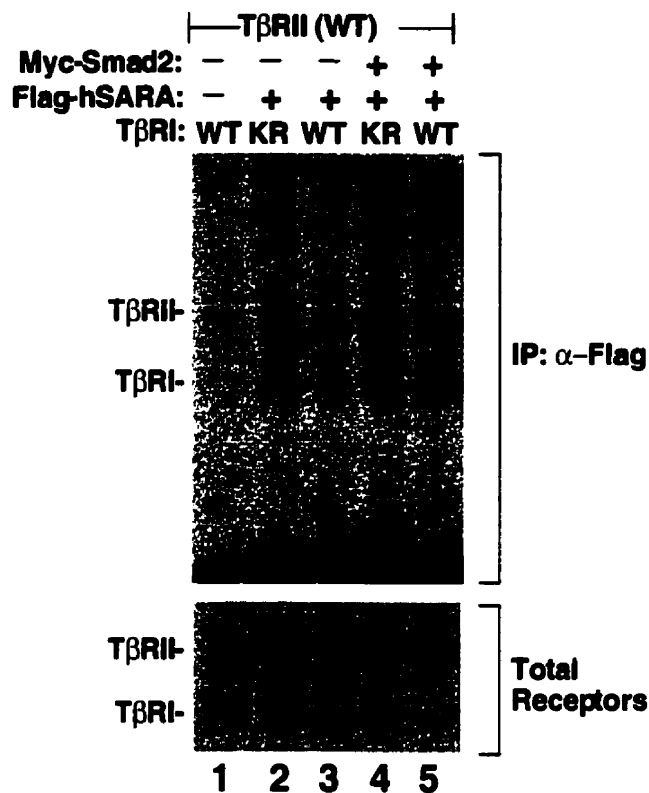


Figure 10. The Association of SARA with TGF- β receptor complexes.

COS cells transfected with both the type I and type II receptors were incubated with [125 I]-labelled TGF- β to induce the formation of an activated receptor complex. Co-expression of SARA in these cells followed by α flag-SARA immunoprecipitation revealed the association of SARA with receptor complexes (lanes 2 & 3). This association was enhanced in the presence of Smad2 (lane 4 & 5). T β RI (K-R) is a kinase deficient receptor which traps the Smad2 substrate, and thus the observed enhancement of SARA-receptor association in the presence of Smad2 indicates a possible cooperative effect. The bottom panel shows that equivalent levels of [125 I]-radioactive receptor complexes were formed in each transfectant.

but is stabilized by kinase deficient T β RI-(KR). Further, these results suggest that SARA associates with the TGF- β receptor complex, and that Smad2 can cooperate with SARA to enhance this association.

IV. The Function of SARA-mediated Localization of Smad2

SARA Δ 1-664 mislocalizes Smad2

The characterization of SARA thus far suggested that it functions to recruit and anchor Smad2 in specialized subcellular regions of the cell where receptors colocalize upon ligand activation. The SARA-mediated localization of Smad2 is thus regulated in essence by two modular domains; the SBD, which interacts with Smad2, and the FYVE finger, which is responsible for subcellular localization. Consequently, the SARA deletion mutant, Δ 1-664 which interacts with Smad2 but fails to localize properly would be useful to address the significance of Smad2 localization for TGF- β signalling. In cells expressing Δ 1-664 and Smad2, both proteins displayed a diffuse distribution throughout the cytosol, unlike in cells expressing wild type SARA (Figure 11A). This suggests that Δ 1-664 fails to recruit Smad2 to punctate domains of the cells and may actually cause the mislocalization of Smad2. To examine this directly, I tested whether Δ 1-664 could mislocalize Smad2 in the presence of wild type SARA. For this, the mutant was coexpressed in Mv1Lu cells in the presence of Smad2 and wild type SARA (Figure 11B). In these cells, while wild type SARA localized to punctate domains as shown in Figure 7D, the localization of Smad2 was disrupted. Thus, SARA (Δ 1-664) can induce the mislocalization of Smad2.

SARA Δ 1-664 abolishes TGF- β Signalling

A SARA mutant that can cause Smad2 to mislocalize provided a useful tool to determine whether anchoring Smad2 in correct subcellular compartments may be a critical

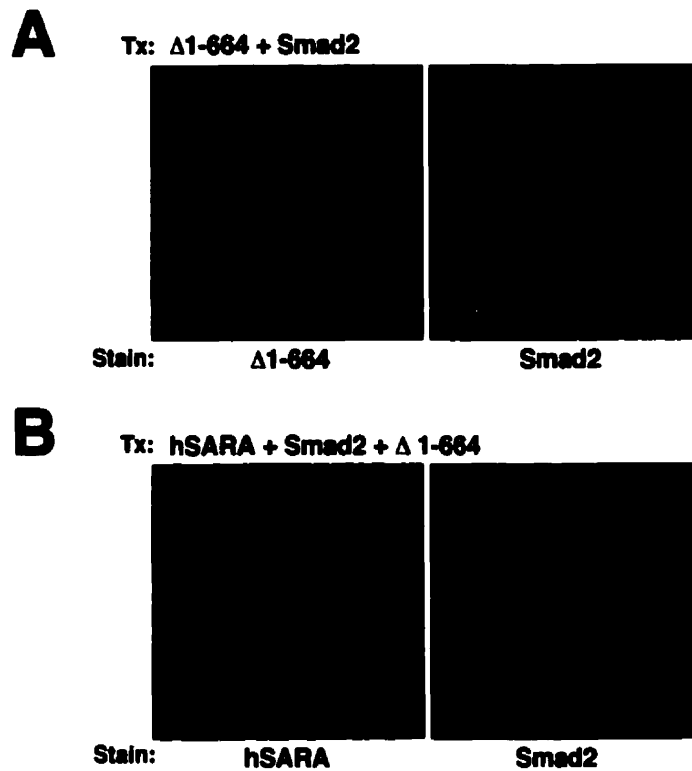


Figure 11. Mislocalization of Smad2 by the SARA mutant $\Delta 1-664$. Mv1Lu cells were transfected with Smad2 and a mutant form of SARA ($\Delta 1-664$) in the absence (A) or the presence (B) of the wild type SARA. Subcellular localization of was analyzed by immunofluorescence and confocal microscopy. The SARA mutant $\Delta 1-664$ lacks the FYVE-finger but retains a functional SBD. Panel A displays the inability of this mutant to recruit Smad2 into the SARA punctate domains. In Panel B, coexpression of this mutant in the presence of wild type SARA continues to mislocalize Smad2 from punctate domains. This result shows that SARA ($\Delta 1-664$) can function to mislocalize Smad2. (Stain: Panel A: α myc- $\Delta 1-664$, α HA-Smad2; Panel B: α myc-SARA, α HA-Smad2 [flag- $\Delta 1-664$ was not stained])

event in TGF- β signal transduction. To investigate this, signalling assays measuring TGF- β transcriptional responses were performed in Mv1Lu cells using the 3TP-Luciferase reporter construct. To compare the effect of Δ 1-664 with wild type SARA, cells transfected with increasing amounts of DNA were tested for TGF- β responsiveness (Figure 12). Expression of wild type SARA had no effect on TGF- β signalling, however Δ 1-664 reduced the TGF- β signal even at the lowest DNA concentration and abolished the signal completely at higher levels. Interestingly, the SARA mutant Δ 1-704 which lacks a functional SBD does not disrupt TGF- β transcriptional responses suggesting that the inhibitory effect of Δ 1-664 requires the function of the SBD. Therefore mislocalizing Smad2 disrupts TGF- β responses, demonstrating that SARA-mediated anchoring of Smad2 in specialized subcellular domains is important for TGF- β signal transduction. (This experiment was performed by Dr. Tomoo Tsukazaki)

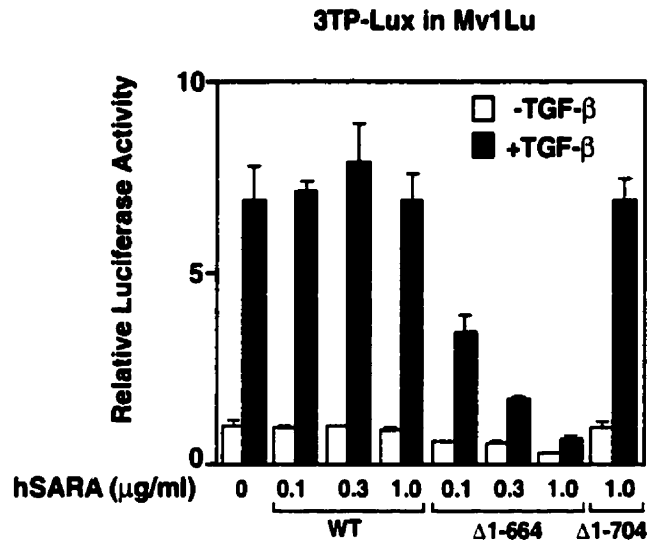


Figure 12. SARA Δ 1-664 abolishes TGF- β transcriptional signalling responses. Mv1Lu cells were transfected with p3TP-Lux alone, wild type SARA, Δ 1-664, or Δ 1-704. For wild type SARA and Δ 1-664, increasing amounts of DNA were examined. Cells were incubated overnight in the absence (open bars) or presence of 100 pM TGF- β (closed bars), and relative luciferase activity was measured in the cell lysates. In cells expressing wild type SARA transcriptional responses were equivalent to reporter signalling (ie. no SARA transfected) whereas expression of the Δ 1-664 mutant reduced this signalling and completely abolished it at higher levels of DNA. SARA Δ 1-704 fails to reproduce the Δ 1-664 effect (see Figure 3), indicating that the inhibitory effect must contain a functional SBD. This result demonstrates that mislocalizing Smad2 by Δ 1-664 abolishes transcriptional signalling responses. p3TP-lux is a TGF- β responsive reporter construct. (This experiment was performed by Tomoo Tsukazaki.)

Chapter 3

Discussion

SARA is essential for TGF- β Signal Transduction

Discussion SARA is essential for TGF- β Signal Transduction

Signal Transduction through Anchoring Proteins

Signal Transduction is the process by which extracellular signals are relayed from the cell surface to specific intracellular sites. The organization and specificity within a signal transduction pathway is achieved through a number of mechanisms that can include controlling the accessibility of enzymes to their respective substrates (Pawson and Scott., 1997). Thus, one mechanism to assemble and control the activity of components of a pathway is through compartmentalization, which can be achieved through the recruitment of signalling proteins to certain subcellular environments. A class of regulatory molecules involved in such a role is known as anchoring proteins (Faux and Scott., 1996). These molecules exist in a signalling network to maintain and localize proteins to their sites of action (targeting loci) through the association with structural proteins, components of membranes, the cytoskeleton or cellular organelles.

Targeting proteins such as anchors, function in essence through two domains: (1) a domain responsible for binding cellular components, and (2) a unique targeting domain that tethers the complex to specific subcellular structures (Faux and Scott., 1996). For example in PKA anchoring, a number of AKAPs (A-Kinase Anchoring Proteins) have been identified, and they function through two domains, one to bind PKA and another to localize it to specific intracellular sites. PKA-AKAP complexes have been identified at the centrosomes, ER, golgi, microtubules, mitochondria, membranes, nuclear matrix and secretory granules (Hausken and Scott, 1996). Therefore, the role of anchoring PKA to precise intracellular sites serves two functions: (1) to expose pools of anchored PKA to gradients of its activator, cAMP; and (2) to ensure compartmentalization and accessibility to its substrates (Mochly-Rosen., 1995).

SARA, an novel Anchor in TGF- β Signalling

In this thesis, my work initiated first to identify a human homolog of *Xenopus* SARA, and secondly to characterize its functional role in the TGF- β signal transduction pathway. As a result, a novel component of this pathway was identified and revealed to function as an important regulator of Smad2 localization. Based on my findings, which I have presented in this thesis, and those done in collaboration with Dr. Tsukazaki, we call this molecule SARA for Smad Anchor for Receptor Activation.

The identification of SARA revealed several inherent qualities based on the primary sequence. First, SARA is not related to any mammalian family of proteins, and thus may potentially define a novel class of Smad interacting proteins. The fact that it interacts with TGF- β / Activin-regulated Smad2 and Smad3, but not with BMP-regulated Smad1, strongly suggests that other structurally related SARA-like proteins may exist to regulate other Smads. The second observation revealed in the SARA sequence is the presence of a domain known as a FYVE-finger. A search in the literature revealed that FYVE-fingers are structurally conserved zinc-finger domains that exist in a number of unrelated molecules (a list of FYVE-finger proteins is presented in Gaullier et al., 1998).

The characterization of SARA-Smad2 interactions demonstrated two important features about SARA function in Smad2 signalling. The first feature is the presence of a novel modular domain now called the **SBD** which dictates the specificity of the SARA-Smad interaction (Cohen et al., 1995). The Smad Binding Domain is necessary and sufficient for interaction with Smad2, implicating the importance of specialized protein modules in mediating the formation of SARA-Smad2 signalling complexes. The second feature is the demonstration that SARA-Smad2 interactions are regulated by phosphorylation events (Hunter, 1995). Specifically, receptor kinase phosphorylation of Smad2 induces dissociation of SARA-Smad2 complexes and the formation of Smad2-Smad4 complexes. This evidently places SARA in an upstream role in the signalling cascade.

The observation that SARA interacts with inactive Smad2 prior to signal activation by receptor kinase implicates the involvement of SARA in two upstream functions: (1) to recruit unphosphorylated Smad2 to sites of activation, and (2) to maintain and facilitate Smad2 activation by TGF- β receptors. Studies of SARA-Smad2 subcellular localization support the notion that Smad2 is recruited to SARA punctate microdomains. In addition, these studies revealed the necessity of the FYVE-finger in targeting SARA complexes to these punctate microdomains. In support of SARA's second upstream function is the finding that the TGF- β receptor colocalizes with SARA punctate domains, and furthermore physically associates with SARA and SARA-Smad2 complexes.

Thus, based on my biochemical characterization and subcellular studies, the role of SARA is to function as an anchor to recruit Smad2 into specialized intracellular sites for receptor activation. SARA possesses two important qualities for being characterized as an anchoring protein; (1) it has the capacity of binding a signalling molecule through a protein module (SBD), and releasing it after activation; and (2) it contains a targeting domain (FYVE-finger) responsible for recruiting the complex to a specific subcellular site. Thus, the SBD and the FYVE-finger are two domains in SARA that function to anchor Smad2 for activation. Having defined a novel anchoring protein in TGF- β signal transduction, the question of functional relevance is examined: Specifically, is Smad2 anchoring by SARA essential for TGF- β signalling? The use of SARA mutants that retain their ability to interact with Smad2, but fail to localize properly allowed this point to be addressed. In particular, the ability of SARA Δ 1-664 to mislocalize Smad2 and abolish TGF- β stimulated transcriptional responses strongly suggests that Smad2 anchoring by SARA in punctate microdomains is essential for TGF- β signal transduction.

Functional Significance of SARA Anchoring

In a cellular environment where signalling events are abundant, the integrity of any single signalling cascade must be tightly regulated. In Smad2 signalling, this regulation is partly accomplished through the mechanism of SARA anchoring which in effect assembles TGF- β signalling components, ie. the receptor kinase T β RI, and its substrate Smad2, at specific intracellular sites of action. Thus, bringing together enzymes and substrates provides an effective and efficient mode of transmitting signals especially if signal inducers, such as receptor kinases exist at low levels (Dyson and Gurdon., 1998), and only initiate signal activation for short periods of time (Green and Smith., 1991 and Gurdon et al., 1995). Pools of anchored Smad2 must be poised and ready for signalling events. SARA anchoring therefore, contributes to the specificity and organization of efficient signal transmission in TGF- β signalling.

Future Directions

Further characterization of SARA function is required in order to better understand its role in TGF- β signalling. Among these is the mechanism of FYVE-finger recruitment. The FYVE-finger is a conserved double zinc finger domain, recently shown to bind the inositol lipid, PtdIns(3)P with high specificity (first shown by Burd et al., 1998; Patki et al., 1998; Gaullier et al., 1998; and Simonsen et al., 1998). Thus, the interaction of the FYVE-finger with membrane phospholipids provides a method for proteins to interact directly with cellular structures containing membranes. Although the FYVE-finger of SARA has yet to be demonstrated to bind PtdIns(3)P, it is likely to function similarly in associating with membrane structures enriched with PtdIns(3)P. Interestingly, the FYVE domain containing proteins Hrs, EEA1, Fab1, Vac1, Vps27 are all involved in controlling of endosomes or vacuole function (Komada et al., 1997; Stenmark et al., 1996; Yamamoto et al., 1995; Piper et al., 1995 and Weisman et al., 1992). Although not all FYVE-finger proteins have been fully characterized in terms of

subcellular localization, the mammalian proteins, Hrs and EEA1 are both localized to early endosomes. Intriguingly, Hrs does not require the FYVE-domain for proper localization whereas EEA1 and SARA do (Mu et al., 1995; Komada et al., 1995 and Stenmark et al., 1996). However, in the light of recent demonstration that the FYVE finger of both EEA1 and Hrs bind PtdIns(3)P specifically (Gaullier et al., 1998), this discrepancy can possibly be resolved by suggesting that other regions of Hrs may be responsible for endosomal targeting. In fact, Simonsen et al., (1998) report that EEA1 contains regions outside of the FYVE that bind proteins localized at early endosomes, namely the GTPase Rab5. Taken together, these observations implicate that SARA may also possess other regions important for additional protein interactions. The concept of a protein with dual binding specificity, as in the case of EEA1, would provide a nice mechanism to ensure proper localization and function (reviewed in Wiedemann and Cockcroft, 1998).

Based on these findings and observations, a number of future lines of investigations can be laid out. First, studies are required to determine the exact nature of the SARA punctate domains. This can be achieved by fluorescent microscopy and the use of a variety of subcellular markers (These studies are currently under intensive examination and preliminary data suggests that SARA may colocalize with EEA1; Anne Davison, unpublished data). Secondly, it would important to confirm whether the FYVE-finger of SARA can bind PtdIns(3)P in a specific manner as demonstrated with EEA1 and Hrs. The demonstration of FYVE-fingers binding PtdIns(3)P, a PI(3)Kinase product, and the observation that EEA1 activity is regulated by this enzyme, raises the possibility that PI(3) Kinase may also regulate SARA activity through its FYVE-finger. Thirdly, inspection of the SARA sequence reveals the presence of two large regions potentially important for additional protein interactions; an amino-terminus portion upstream of the SBD, and a carboxy-terminus region downstream of the FYVE-finger. As an anchoring molecule, these two regions or portions within them may serve as

interaction domains that bind other proteins or possess some other unidentified function. It is possible that SARA may function as an anchored signalling scaffold, and thus it would be interesting to define other proteins that interact with SARA.

Taken together, these results lead me to propose the following hypothesis for the function of SARA (Figure 13).

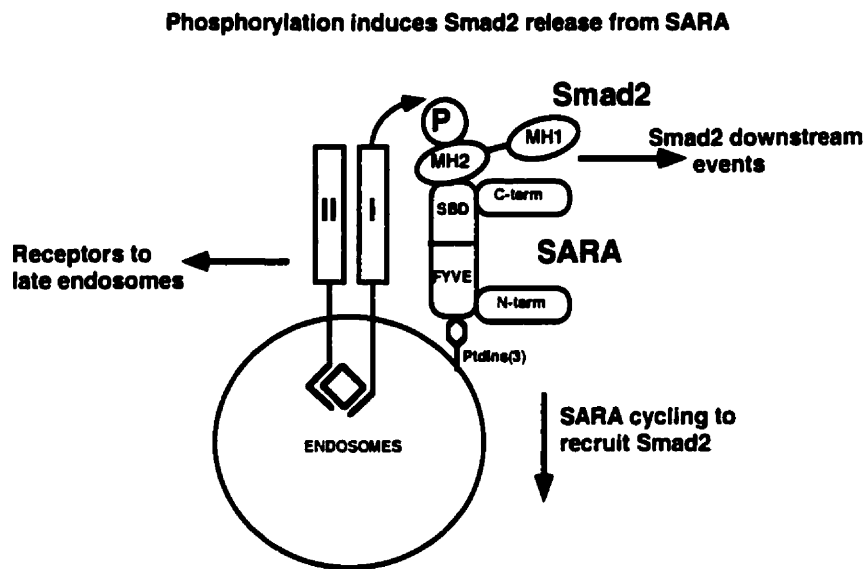


Figure 13. The Hypothetical Signalling Model of Smad Anchoring for Receptor Activation. The formation of an activated receptor complex results in the internalization to endosomal vesicles where SARA is anchoring Smad2 for efficient phosphorylation. The receptor activation of Smad2 results in the release of Smad2 from these sites and allows it to mediate its downstream functions.

TGF- β receptor activation results in the internalization of the receptor kinase complexes to early endosomes. Although the mechanisms of TGF- β receptor endocytosis is still unclear and currently under study, (Anders et al., 1997 and Anders et al., 1998) other signalling pathways have evidence that internalized receptors continue to signal from within the endosome (reviewed in Baass et al., 1995 and reports in Vieira et al., 1996 and Di Guglielmo et al., 1998). Thus, it can be postulated that SARA anchoring complexes are localized on the cytoplasmic surface of early endosomes where it holds the receptor substrate, Smad2 for phosphorylation by the internalized receptor

complex. Serine phosphorylation activates Smad2 and releases it from SARA. This event would initiate the propagation of downstream signalling events such as the formation of Smad2-Smad4 complexes which translocate to the nucleus and regulate specific target genes. Conceivably, the release of Smad2 from SARA could also stimulate the vesicular transport of TGF- β receptor complexes from early endosomes to late endosomes and lysosomes for degradation or to an alternative transport pathway where receptors are recycled back to the cell surface. This may provide an important pathway to turnoff TGF- β signalling. The mechanism of this process may possibly be regulated by Rab proteins (reviewed in Novick et al., 1997). Finally, SARA may also function in cycling the recruitment of Smad2, and once again prepare it for receptor activation. This would provide a necessary step for future TGF- β restimulation.

And thus, undoubtedly it would be of great interest and excitement to see what awaits ahead in the future work of SARA function in TGF- β signal transduction.

Chapter 4

Materials and Methods

Materials and Methods

Cloning of SARA

In order to clone the human homolog of the *Xenopus* SARA, I employed a PCR strategy using primers designed with degenerate nucleotides based on the amino acid sequence of xSARA. The forward primer was chosen in the FYVE-domain whereas the reverse primer was designed from the Smad Binding Domain. The amino acids within these two important domains are likely to be conserved and identical between species, and therefore increase the chance of identifying the homolog. The sequence of the forward and reverse primers are:

5'--GC(a/c/g/t)CC(a/c/g/t)AA(c/t)TG(c/t)ATGAA(a/c/g/t)TG(c/t) --3' and

5'--(a/g)CA(a/g)TA(c/t)TC(a/c/g/t)GC(a/c/g/t)GG(a/g)TT(a/g)TT --3'.

The template for the PCR reactions was derived from RNA that was isolated from the human liver carcinoma, HepG2. Total RNA was isolated using the Trizol RNA reagent according to the manufacture's recommendations (Gibco/BRL). To synthesize cDNA, the RNA was subjected to reverse transcription using random hexamers as the primers (Perkin Elmer). The degenerate PCR reaction was first subjected to 3 cycles of amplification at the annealing temperature of 42 °C, followed by 30 cycles of amplification with annealing at 55 °C (as described in Attisano et al., 1992). The resulting PCR product was approximately 250 bases long as predicted, and was ligated into pCMV5 and transformed into DH5 α . 25 individual bacterial clones were isolated and sequenced. The sequence of positive clones was compared at the amino acid level to xSARA and confirmed to be highly conserved (94% identity). This DNA fragment was reamplified by PCR in the presence of [³²P]-radiolabelled dATP and used as probe to screen a λ ZAP phage human fetal brain cDNA library (Stratagene). The cloning procedures to isolate positive plaques were carried out according to protocols described by the manufacturer (Stratagene). Eight positive plaques were isolated, and their cDNAs

were characterized by restriction enzyme digestion. Two out of the eight clones produced identical restriction maps and sequence comparison of one of them to xSARA revealed 86% identity in the FYVE domain and SBD, however it was truncated at the carboxy terminus. Searches in the GenBank EST database using the sequence of this truncated clone resulted in the identification of an EST (EST#183440) that overlapped with my phage clone and encoded the downstream 3' region. Careful sequence analysis demonstrated that my clone and the EST clone were identical in a region that overlapped by approximately 1 Kb. A full length clone covering the entire open reading frame was generated as a fusion of the two clones at the *Stu*I restriction enzyme site. To verify the open reading frame, the 5'UTR of SARA was extended by sequencing another overlapping EST clone (EST#260739) and led to the identification of stop codons in all three reading frames. The subsequent ORF of SARA is 1323 amino acids.

In vitro SARA Interactions

The in vitro transcription and translation of full length SARA was produced using the TNT coupled reticulocyte lysate system in accordance to the manufacturer's protocols (Promega). Briefly, transcription reactions employed the use of the T3 RNA polymerase, whereas translation utilized the cellular machinery and components of rabbit reticulocytes. Production of SARA protein was carried out in the presence of [³⁵S]-methionine to label the product. GST fusion proteins were produced in bacteria transformed with the appropriate pGEX fusion constructs. Briefly, 1 ml of overnight culture was inoculated into 9 ml of LB media and protein expression was induced by adding IPTG to a final concentration of 0.1 mM. Cultures were grown for an induction period of 3 hrs at 37 °C, and bacterial cells were lysed by sonication in TNTE buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100 and 1 mM EDTA). The expressed fusion proteins were purified by incubating bacterial lysates with glutathione sepharose beads. Protein interactions were carried out by incubating radiolabelled SARA with GST

proteins immobilized on beads for 2 hours at 4 °C. Beads were washed five times in TNTE (same as above, except with 0.1% Triton X-100), and bound proteins were separated by SDS-PAGE and visualized by fluorography. Total protein expression of GST-fusion proteins were quantitated by coomassie staining.

Construction of SARA Deletion Mutants

The generation of SARA constructs was accomplished by common subcloning strategies. All SARA constructs except for GST-SBD were subcloned in the mammalian expression vector, pCMV5 containing an amino terminal Flag epitope (Hoodless et al., 1996). SARA mutants, Δ 1-344, Δ 1-892, Δ 893-1323, Δ 346-1323, and Δ 597-706 were constructed by deletion of Sall-XbaI, Sall-EcoRV, EcoRV-HindIII, XbaI-HindIII and Asp718-Asp718 fragments, respectively. SARA mutants, Δ 1-594 and Δ 1-704 were constructed by partial digestion using Sall-Asp718, whereas Δ 665-1323 was constructed by partial digestion using Asp718-HindIII. The remaining SARA mutants, Δ 1-531, Δ 1-664, Δ 750-1323, Δ 596-1323, Δ 665-704, Δ 597-664 and the fusion construct pGEX4T-1;GST-SBD (aa 665-750) were constructed by PCR using appropriate primers and convenient restriction sites. The preparation of SARA constructs was done in collaboration with Dr. Tomoo Tsukazaki.

In vivo SARA Interaction Studies

All SARA biochemical interaction studies were done in COS cells. DNA transfections were performed using LipofectAMINE according to the manufacturer's protocols (Gibco/BRL). 44-48 hours after transfections, cells were lysed (as described in Wrana et al., 1994) and the lysates subjected to incubation with Flag antibodies (Sigma) at 4 °C for 1 hour. Antibody-protein complexes were immunoprecipitated by adsorption to protein-G sepharose beads. Bound proteins on beads were washed, separated by SDS-PAGE, and transferred to nitrocellulose membranes (as described in

Attisano et al., 1996). Western blotting of membranes were incubated with the appropriate primary monoclonal antibodies: Myc (9E10), Flag (Sigma) or HA (12CA5, Boehringer Mannheim), followed by a goat anti-mouse secondary antibody covalently linked to horse radish peroxidase. Immune complexes on membranes were visualized by ECL (Amersham Life Science) and autoradiography. As mentioned in the second chapter, the *in vivo* interaction experiment shown in Figure 4 was performed by Dr. Tomoo Tsukazaki.

The association of SARA with TGF- β receptors were performed on transiently transfected COS cells by affinity labelling cells with 200pM [125 I] TGF- β in media containing 0.2% bovine fetal serum at 37 °C for 30 min (Macías-Silva et al., 1996). Radioactive receptor complexes were stabilized by crosslinking [125 I] TGF- β complexes with DSS for 15 min at 4 °C (Massagué, 1987). The cells were then lysed and protein interactions were visualized by immunoprecipitation with antibodies directed to the Flag-tag on SARA as described above. Affinity labelled TGF- β receptor complexes present in SARA immunoprecipitates were then resolved by SDS-PAGE, and visualized by autoradiography using BioMax film (Kodak BioMax, MS).

SARA-SBD and Smad2 Pulldown Assay

The GST-SBD interaction assays with Smad2 were done by incubating immobilized GST-SBD prepared from bacteria (as described in **In vitro SARA Interactions**) with COS cell lysates prepared from cells expressing Smad2 (transfected as described above in **In vivo SARA Interaction Studies**). Bound Smad proteins on GST-SBD pulldowns were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting to detect Smad2 was carried out in a similar fashion as described above. Total protein expression of GST-SBD was quantitated by coomassie staining.

SARA Immunofluorescent Confocal Microscopy

Mv1Lu cells plated in chamber glass slides (Nalgene Nunc) were transiently transfected with the construct(s) of interest using the CaPO₄ method of transfection. 14-16 hours after DNA-CaPO₄ precipitate addition, cells were washed with fresh media (MEM + NEAA + 10 % FBS) and incubated for another 24 hours before fixing and staining. In the case where ligand stimulation was required, TGF-β (200pM final concentration) was added to the media containing 0.1% bovine serum, 1 hour prior to fixation and staining. Fixation was accomplished by incubating cells in 4% paraformaldehyde (prepared freshly by diluting a 20% stock) for 10 min at room temperature. Fixed cells were then washed with PBS five times and permeabilized by incubation in 100% methanol for 2 min at room temperature. After permeabilization, cells were washed in PBS for five times, and incubated with PBS containing 10% goat serum for 1 hour at room temperature to block non-specific staining. Following blocking, the cells were incubated with the appropriate primary antibodies in the same solution (PBS + 10% goat serum) for 4 hours at room temperature. For the SARA staining with Smad2, I used the myc-A14 rabbit polyclonal (Santa Cruz) at a final concentration of (1μg/ml) to detect Myc-SARA and Flag-M2 monoclonal (Sigma) at 10 μg/ml to detect Flag-Smad2 (Figure 7). For the localization of SARA deletion mutants, Flag-tagged constructs were also stained with Flag-M2 at 10 μg/ml (Figure 8). For the analysis of SARA localization with TβRII receptor, TβRII-HA was stained with HA-12CA5 monoclonal (Boehringer Mannheim) at 10μg/ml, and Myc-SARA was stained as before (Figure 9). The mislocalization study with Δ1-664 were carried out with Myc-Δ1-664 and HA-Smad2 in panel A, and with Myc-SARA, HA-Smad2 and Flag-Δ1-664 in panel B. Primary antibodies used in this study were with Myc-A14 polyclonal and HA-12CA5 monoclonal at concentrations mentioned above; no Flag staining was done (therefore Flag-Δ1-664 in panel B is not stained). After incubation with primary antibodies five washes with PBS were carried out, and the appropriate fluorescent

conjugated secondary antibodies were added to cells for 1 hour in the dark at room temperature. I used FITC-conjugated goat anti-mouse, and Rhodamine-Texas Red-conjugated goat anti-rabbit for detection of monoclonal and polyclonal antibodies, respectively, at a 1:200 dilution (Jackson Laboratories). All cells were then washed again in PBS five times and then counter stained for nuclei using DAPI (Sigma) at a final concentration of 1mg/ml in PBS for 5 min. Cells were mounted with mowiol-DABCO (Mowiol 4-88 (Hoechst), glycerol, Tris-HCl, 1,4-diazobicyclo-[2.2.2]-octane (DABCO); see Harlow et al., 1988 for exact procedure). Immunofluorescent confocal analysis was performed using a Leica Confocal microscope.

TGF- β Transcriptional Response Assay

The 3TP-Luciferase construct is a TGF- β transcriptional responsive reporter (Wrana et al., 1992). Mv1Lu cells were transiently transfected in triplicates with this reporter, a CMV- β -gal reporter, and the indicated SARA constructs using the DNA-CaPO₄ coprecipitation method. The amount of DNA (μ g/ml) indicated at the bottom of the graph (Figure 12) were used for 3 wells in a 24-well plate. 14-16 hours after addition of the precipitate, cells were washed and subsequently incubated overnight with TGF- β (at a final concentration of 100pM) in media containing 0.1% fetal bovine serum. On following day the cells were lysed, and an aliquot of the lysate was analyzed for luciferase activity by incubating with the luciferin substrate. The light output was quantitated with a Berthold Lumat LB luminometer. To normalize for transfection efficiency, a second aliquot of the cell lysate was incubated with the β -gal substrate ONPG, and the relative galactosidase activity was measured using a spectrometer (Molecular Devices, ThermoMax microplate reader, see Maniatis et al., 1989). Relative luciferase activity was calculated by normalizing luciferase activity readings to galactosidase activity present in each transfectant and are plotted as the mean \pm standard deviation.

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