Characterization of the Smad Anchor for Receptor Activation in TGFβ Signal Transduction

by

Anne Frances Davison

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

©Copyright by Anne Frances Davison (2000)
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-50428-X
Characterization of the Smad Anchor for Receptor Activation
In TGFβ Signal Transduction

By: Anne Frances Davison

Master of Science, 2000
Graduate Department of Molecular and Medical Genetics,
University of Toronto

ABSTRACT

A novel essential component of the Transforming Growth Factor beta (TGFβ) signaling pathway that is referred to as SARA, for Smad Anchor for Receptor Activation, has recently been identified (Tsukazaki, T. et al. 1998). Preliminary analysis has revealed that SARA recruits Smad2 to the TGFβ receptor complex by controlling its subcellular localization. To further characterize the role of SARA in the signal transduction pathways of the TGFβ superfamily, I have undertaken a detailed analysis of this protein. I have shown that SARA is ubiquitously co-expressed with Smad2 in adult human tissues. In addition, dual label immunofluorescence confocal microscopy analysis has demonstrated that SARA localizes specifically to early endosomes, and that PI(3)P participates in mediating this localization. I have also further characterized the association between SARA and the TGFβ receptors. I have demonstrated that SARA interacts with specific type I receptors, including the activin receptor-like kinase 1 (ALK1), and the TGFβ and activin type I receptors. Moreover, my characterization has revealed that this interaction occurs independent of signaling, and is mediated by the carboxy terminus (amino acids 1000-1323) of SARA. Together, these results lead to a model of SARA functioning as a scaffold for the type I receptor kinase and its substrate, Smad2, in the early endosome.
## TABLE OF CONTENTS

ABSTRACT                                             i  
ACKNOWLEDGEMENTS                                      ii 
TABLE OF CONTENTS                                     iii 
LIST OF FIGURES AND TABLES                            v  
ABBREVIATIONS                                         vi 

1. **INTRODUCTION**                                 1  
   1.1 The TGFβ Signal Transduction Network             1  
   1.1.1 The TGFβ superfamily of serine/threonine kinase receptors 1  
   1.1.2 The Smad protein signal transducers            2  
   1.1.3 Function of Smads in the nucleus               4  
   1.2 hSARA (Smad Anchor for Receptor Activation)      7  
      1.2.1 Cloning of SARA                             7  
      1.2.2 SARA domains                               8  
         1.2.2.1 The FYVE domain                        8  
         1.2.2.2 The Smad Binding Domain                10  
      1.2.3 The role of SARA in TGFβ signaling          10  
   1.3 Current Model for the TGFβ Signaling Pathway     11  
   1.4 Thesis Rationale, Objectives and Hypothesis      11  

2. **MATERIALS AND METHODS**                        13  
   2.1 Cell Culture                                    13  
   2.2 RT-PCR Analysis                                 13  
   2.3 Northern Blot Analysis                          14  
   2.4 Immunofluorescence and Confocal Microscopy      15  
   2.5 Subcloning                                      16  
   2.6 Production and Purification of Glutathion-S-Transferase Fusion Proteins 17  
   2.7 Transient Transfection of DNA into Mammalian Cell Lines 18  
   2.8 Cell Lysate Preparation and Immunoprecipitation 19  
   2.9 SDS-PAGE and Western Blotting                   20  

3. **RESULTS**                                       21  
   3.1 Expression Analysis of SARA                     21  
      3.1.1 RT-PCR analysis of SARA expression in various cell lines 21  
      3.1.2 Northern Blot Analysis                       24  
   3.2 Subcellular Localization of SARA                 24  
   3.3 Characterization of the FYVE Domain of SARA      30  
   3.4 Characterization of Interaction Between SARA and the TGFβ Superfamily of Receptors 34  
      3.4.1 Specificity of interaction between SARA and the TGFβ family of receptors 35
3.4.2 Signaling dependence of interaction between SARA and the type I receptors 38
3.4.3 Mapping of regions in SARA important in mediating interaction with the type I receptors

3.5 Summary of Results 41

4. DISCUSSION 44

4.1 Characterization of SARA-Receptor Association 44
  4.1.1 Specificity of SARA-receptor interactions 44
  4.1.2 Mapping of the receptor binding domain 46
  4.1.3 SARA as a scaffold 46

4.2 Model of SARA at the Early Endosome 47

4.3 Functional significance of SARA 48

4.4 Future Directions 50
  4.4.1 Further defining SARA-type I receptor association 50
  4.4.2 Identification of novel SARA interacting proteins 51

5. APPENDIX 1: Analysis of Involvement of Hrs/Hgs FYVE Domain Protein in the TGFβ Signaling Pathway 54

5.1 Introduction 54
  5.1.1 The Hgs FYVE domain protein 54
  5.1.2 Rationale and objectives 55

5.2 Results 56
  5.2.1 Analysis of subcellular localization of Hgs with respect to SARA 56
  5.2.2 In vivo interaction of Hgs and specific TGFβ superfamily type I receptors 56

5.3 Discussion 62

6. REFERENCES 64
LIST OF FIGURES AND TABLES

Figures

Figure 1A Alignment of FYVE domain amino acid sequences 9
Figure 1B Structure of hSARA 9
Figure 2 A model for the initiation of the TGFβ signaling pathway 12
Figure 3 SARA expression analysis in multiple cell lines using RT-PCR 23
Figure 4 SARA and Smad2 expression patterns in different human tissues 26
Figure 5A Subcellular localization of SARA with respect to early endosomal marker, EEA1 29
Figure 5B Subcellular localization of SARA with respect to late endosomal marker, rab9 29
Figure 6A Effect of wortmannin on the subcellular localization of SARA 32
Figure 6B Production and purification of GST-FYVE fusion proteins 32
Figure 7A Interaction of SARA and the type I receptors 37
Figure 7B Interaction of SARA and the type II receptors 37
Figure 8 Signaling dependence of SARA-ALK4 interaction 40
Figure 9A Mapping of the type I receptor binding domain in SARA 43
Figure 9B Further defining the type I receptor binding domain in SARA 43
Figure 9C Summary of SARA deletion mutants used to map the receptor binding domain 43
Figure 10 Proposed model for SARA as a scaffold 49
Figure 11A Subcellular localization of Hgs 58
Figure 11B Subcellular localization of Hgs with respect to SARA 58
Figure 12 Hgs interacts with specific type I receptors 61

Tables

Table 1 Molecular components of the signal transduction pathway of the TGFβ superfamily 5
ABBREVIATIONS

α - anti
aaa - amino acid
actRII - activin type II receptor
actRIIB - activin type II receptor B
ALK1 - activin receptor-like kinase 1
ALK2 - activin receptor-like kinase 2
ALK3 - (BMPR-IA), activin receptor-like kinase 3
ALK4 - (actRIB), activin receptor-like kinase 4
ALK5 - (TβRI), activin receptor-like kinase 5
ALK6 - (BMPR-IB), activin receptor-like kinase 6
ALK7 - activin receptor-like kinase 7
β - beta
BMP - bone morphogenetic protein
bp - base pair
CBP - CREB binding protein
cDNA - complementary DNA
CREB - cyclic AMP response element-binding protein
DEPC - diethylpyrocarbonate
Dpp - decapentaplegic
DNA - deoxyribonucleic acid
dNTPs - deoxynucleoside triphosphates
DSARA - Drosophila SARA
DTT - dithiothreitol
EDTA - ethylenediaminetetraacetic acid
EEA1 - early endosome autoantigen 1
EGF - epidermal growth factor
EGFR - epidermal growth factor receptor
FBS - fetal bovine serum
FGD1 - faciogenital dysplasia gene product
GM-CSF - granulocyte-macrophage colony-stimulating factor
GST - glutathione S-transferase
HA - hemagglutinin epitope
HDAC - histone deacetylase
Hgs - (Hrs) hepatocyte growth factor-regulated tyrosine kinase substrate
HGF - hepatocyte growth factor
hSARA - human SARA
IC₅₀ - inhibitory concentration of 50%
IL-2 - interleukin 2
IPTG - isopropyl-β-D-thiogalactopyranoside
kb - kilobase
kDa - kilodalton
Mad - Mothers against dpp
MAPK - Mitogen-activated protein kinase
MH1 - Mad homology 1
MH2 - Mad homology 2
min - minute
mRNA - messenger RNA
NaCl - sodium chloride
NaF - sodium fluoride
N-CoR - Nuclear hormone receptor corepressor
NEAA - nonessential amino acids
OD - optical density
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PDGF - platelet-derived growth factor
PI(3)K - phosphoinositide 3'-kinase
PI(3)P - phosphatidylinositol 3-phosphate
PKA - protein kinase A
PP1 - protein phosphatase 1
RNA - ribonucleic acid
RT-PCR - reverse transcriptase - PCR
R-Smad - receptor regulated Smad
SARA - Smad anchor for receptor activation
SDS-PAGE - sodium dodecyl sulfate - polyacrylamide gel electrophoresis
sec - second
ser - serine
SH2 - Src homology 2
SH3 - Src homology 3
Ski - Sloan-Kettering Institute
SnoN - Ski related Novel gene
SRC-1 - Steroid Receptor Coactivator-1
STAM - signal-transducing adaptor molecule
Ste - Sterile 5
TβRII - TGFβ type II receptor
TFE3 - transcription factor μE3
TGFβ - transforming growth factor beta
TGIF - 5'TG3' interacting factor
thr - threonine
VDR - vitamin D receptor
XSARA - Xenopus SARA
INTRODUCTION

1.1 The TGFβ Signal Transduction Network

The Transforming Growth Factor-β (TGFβ) superfamily comprises structurally related polypeptide growth factors that exert diverse biological effects on cell growth, differentiation, apoptosis, and early development (reviewed in (Hoodless, P.A. and Wrana, J.L. 1997; Massagué, J. 1998)). In addition, members of this family are involved in cancer (reviewed in (Markowitz, S.D. and Roberts, A.B. 1996; Massagué, J. and Weis-Garcia, F. 1996)). Recent studies have led to the elucidation of the mechanisms and components of a unique signal transduction network that mediates the cellular responses to these growth factors. This network is composed of transmembrane serine/threonine kinase receptors, the intracellular mediators, Smads, and the nuclear targets and DNA binding partners with which Smads associate. The regulation of the intracellular mechanisms of TGFβ signaling transduction is currently of great interest and research effort.

1.1.1 The TGFβ superfamily of serine/threonine kinase receptors

TGFβ and related factors signal through transmembrane receptor serine/threonine (ser/thr) kinase receptors (reviewed in (Lin, H.Y. and Lodish, H.F. 1993; Attisano, L. et al. 1994; Miyazono, K. et al. 1994; Liu, F. et al. 1995; Massagué, J. and Weis-Garcia, F. 1996; ten Dijke, P. et al. 1996). Based on their structure and function, these ser/thr receptors are subdivided into two groups known as the type I and type II receptors. Type I receptors have a higher level of sequence similarity amongst themselves than with the type II receptors, particularly in their kinase domains. In contrast to type II receptors, type I receptors lack a serine/threonine rich tail and possess a highly conserved and functionally important 30 amino acid region that is known as the ‘GS domain’. Both the type I and the
type II class of receptors are required for signaling by members of the TGFβ superfamily (Wrana, J.L. et al. 1992; Franzén, P. et al. 1993; Bassing, C.H. et al. 1994; Cárcamo, J. et al. 1994). In the presence of ligand, the type I and type II receptors form an active heteromeric complex (Wrana, J.L. et al. 1994). Within this receptor complex, receptor II transphosphorylates receptor I on serine/threonine residues in its GS domain (Ventura, F. et al. 1994; Wrana, J.L. et al. 1994). This phosphorylation activates the type I receptor kinase, which results in the propagation of signal to the downstream signal transducers known as Smads.

1.1.2 The Smad Protein Signal Transducers

The founding member of the Smad family was first identified in Drosophila melanogaster, as the product of the gene, Mothers against decapentaplegic (Mad) (Raftery, L.A. et al. 1995; Sekelsky, J.J. et al. 1995). The discovery of Smad proteins has led to the elucidation of the mechanism by which the TGFβ superfamily conveys signals from membrane receptors to the nucleus (reviewed in (Attisano, L. and Wrana, J.L. 1998; Kretzschmar, M. and Massagué, J. 1998; Massagué, J. 1998; Padgett, R. et al. 1998, Wrana, J.L. and Pawson, A. 1997). Thus far, eight mammalian Smad proteins have been identified. On a structural and functional basis, the Smads may be divided into three domains, the highly conserved amino- and carboxy-terminal domains - referred to as Mad Homology 1 (MH1) and Mad Homology 2 (MH2) domains, respectively - and a non-conserved intervening linker region (reviewed in (Heldin, C.-H. et al. 1997; Attisano, L. and Wrana, J.L. 1998; Massagué, J. 1998)). Recently, these domains have been shown to have different functions. The MH2 domain of Receptor regulated Smads (R-Smads) is regarded as the 'effector' domain. For example, when fused to a GAL4 DNA-binding domain, the R-Smad MH2 exhibits transcriptional activity (Liu, F. et al. 1996). In addition, the MH2 is responsible for homomeric and heteromeric interactions with R-Smads and

The Smad family of proteins can be subdivided into three groups based on functional criteria: (1) the receptor-regulated Smads (R-Smads), (2) the common Smad, and (3) the inhibitory Smads (anti-Smads). The R-Smads, which include Smads 1, 2, 3, 5 and 8, are substrates of the type I TGFβ family receptor kinases (Macías-Silva, M. et al. 1996; Kretzschmar, M. et al. 1997). The activated type I receptor phosphorylates the R-Smad on the last two serine residues of a characteristic carboxy-terminal 'SSXS' motif (Abdollah, S. et al. 1997; Kretzschmar, M. et al. 1997; Souchelnytskyi, S. et al. 1997). The interaction between the R-Smads and the type I receptor conveys specificity on the pathway. For example, TGFβ and activin signaling is mediated by Smad2 and Smad3, whereas, the Bone Morphogenetic Proteins (BMPs), a major subgroup of the TGFβ family, signal through Smad1, Smad5, and Smad8. Once phosphorylated, the R-Smads hetero-oligomerize with the common Smad, Smad4, which is required for subsequent nuclear translocation (Lagna, G. et al. 1996; Zhang, Y. et al. 1996; Liu, F. et al. 1997; Wu, R.-Y. et al. 1997; Zhang, Y. et al. 1997). The inhibitory Smads include Smad6, which preferentially inhibits BMP signaling, and Smad7, which inhibits TGFβ and activin signaling. This inhibition is accomplished by blocking the access of the R-Smad to the type I receptor, or by disrupting Smad4-heteromeric complexes (Hayashi, H. et al. 1997; Imamura, T. et al. 1997; Nakao, A. et al. 1997a; Topper, J.N. et al. 1997; Hata, A. et al.
1998). Table 1 summarizes the components of well characterized mammalian TGFβ superfamily signaling cascades.

1.1.3 Function of Smads in the Nucleus

Upon translocation to the nucleus, Smad protein complexes play an important role in the regulation of target gene expression. This nuclear function was first predicted from studies demonstrating that the MH2 domains of Smad2 and Smad4 have transcriptional activity when fused to a heterologous DNA binding domain (Liu, F. et al. 1996). In addition, Smads have been shown to participate in transcriptional complexes. These complexes can bind to Smad-responsive promoter DNA directly through Smad-binding elements (Kim, J. et al. 1997; Zawel, L. et al. 1998; Dennler, S. et al. 1998; Jonk, L.J. et al. 1998; Stroschein, S.L. et al. 1999a). Smads are able to achieve highly specific regulation of target promoters through physical or functional interaction with nearby bound transcription factors, for example, 1) Smad2/Smad4 forms a complex with FAST1 that is prebound to the activin response element of the Xenopus Mix.2 promoter (Chen, X. et al. 1996; Chen, X. et al. 1997), 2) Smad2/Smad4 associate with FAST2 that is prebound to the mammalian goosecoid promoter activin response element (Labbé, E. et al. 1998), 3) the Smad3/Smad4/c-Jun/c-Fos complex interacts with TGFβ responsive elements in the collagenase promoter (Zhang, Y. et al. 1998), 4) the Smad3/Smad4/AP-1 complex binds to elements of the c-Jun promoter (Wong, C. et al. 1999), 5) the Smad3/Smad4/TFE3 (transcription factor ßE3) complex interacts with TGFβ responsive elements in the human plasminogen activator inhibitor-type 1 gene promoter (Hua, X. et al. 1998), 6) Smad3 and Sp1 synergistically regulate the human p21 promoter (Moustakas, A. et al. 1998) and 7) polyomavirus enhancer binding protein 2 (PEBP2) α subunits form a complex with Smad3 and cooperatively stimulate transcription of the germline Ig Cα promoter (Hanai, J-i. et al., 1999).
Table 1. Components of the pathways of the TGFβ superfamily. The individual components of the relatively well characterized signaling cascades of the TGFβ superfamily are shown.
Interestingly, Smads may also act to mediate cross talk between TGFβ signaling and other pathways. For example, Smad3 potentiates ligand-induced transactivation of the vitamin D receptor as a coactivator in a complex with VDR and a member of the steroid receptor coactivator-1 (SRC-1) protein family in the nucleus (Yanagisawa, J. et al. 1999). In contrast, Smad7 negatively regulates VDR function through abrogation of this Smad3-mediated VDR potentiation by inhibiting the formation of the VDR-Smad3 complex (Yanagi, Y. et al. 1999).

To regulate the transcriptional activity of TGFβ responsive genes, Smads recruit transcriptional coactivators and corepressors to the promoters of these genes. For example, Smads interact with p300/CBP (CREB Binding Protein) coactivators to mediate transcriptional activation events (Feng, X.-H. et al. 1998; Janknecht, R. et al. 1998; Pouponnot, C. et al. 1998; Topper, J.N. et al. 1998). Alternatively, activated Smads may recruit corepressors such as TGIF (5’TG3’ interacting factor) and histone deacetylases (HDACs) to Smad target promoters, repressing transcription (Wotton, D., et al. 1999a; Wotton, D., et al. 1999b). Recently, the oncoproteins Ski (Sloan-Kettering Institute) and SnoN (Ski-related Novel gene) have been identified as Smad corepressors (Luo, K. et al. 1999; Stroschein, S.L. et al. 1999b). Ski and SnoN repress the ability of Smads to activate transcription through recruitment of nuclear hormone receptor corepressor (N-CoR) and possibly its associated HDAC. Smad transcriptional activity may also be repressed by inhibiting its DNA binding ability; for example, the nuclear oncoprotein Evi-1 interacts with Smad3, thereby preventing Smad3 from binding DNA and blocking TGFβ-induced growth arrest in some cell types (Kurokawa, M. et al. 1998). Thus, Smads function in higher order DNA binding complexes to regulate specific target gene expression in a ligand dependent manner. The effects of this gene regulation ultimately give rise to the cellular responses of the TGFβ superfamily of growth factors.
1.2 hSARA (Smad Anchor for Receptor Activation)

1.2.1 Cloning of SARA

A Smad2 interacting protein of 1235 amino acids was identified by an expression library screen carried out with the Smad2 MH2 domain. Based on subsequent analysis, this clone was named XSARA, for *Xenopus* Smad Anchor for Receptor Activation (Tsukazaki, T. *et al.* 1998). The human homolog of XSARA was cloned in order to analyze the function of SARA in TGFβ signaling in mammalian cells. A 1323 amino acid protein that is 62% identical with XSARA was identified using degenerate RT-PCR and a human brain cDNA library screen. Amino acid sequence comparison revealed that XSARA and the human clone are 35% identical in their amino-terminal regions (amino acids (aa) 1-570) and 85% identical in their carboxy-terminal regions (aa 571-1323). Based on this sequence similarity and subsequent functional characterization, this clone was designated the human SARA homolog, hSARA. In addition, a *Drosophila melanogaster* clone with a carboxy terminus that is 54% identical to the hSARA carboxy-terminus (aa 587-1323), was recently identified in the GenBank database. Similar to XSARA and hSARA, this clone interacts with Smads 2 and 3 (Teresa Reguly, personal communication) and has been designated DSARA. Interestingly, unlike *Xenopus* and human SARA, DSARA binds to other Smads and may have alternative functions. GenBank database searches have revealed that hSARA, XSARA and DSARA are not part of any known protein family. Thus, SARA is evolutionarily conserved and defines a new family of proteins.
1.2.2 SARA Domains

1.2.2.1 The FYVE domain

A conserved double zinc finger, known as the FYVE domain, is found in the central portion of SARA (aa 587-655 in hSARA, aa 510-578 in XSARA and aa 521-589 in DSARA). Amino acid sequence comparison reveals that the FYVE domains of the human, Xenopus, and Drosophila SARA clones are highly conserved (Figure 1). Specifically, hSARA and XSARA FYVE domains are 96% identical while hSARA and DSARA FYVE domains are 57% identical. The FYVE finger module, which is named after the first letter of the first four proteins found to contain it (Fablp, YOTB, Vac1p and EEA1), is a double-zinc binding domain that is conserved in more than 30 proteins of diverse function. Numerous yeast proteins implicated in vesicular transport, such as Vps27p, Fablp, and Vac1p, contain FYVE domains. In addition, the FYVE finger module is found in mammalian proteins such as hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs or Hgs), and early endosome autoantigen 1 (EEA1) (reviewed in Wiedemann, C. and Cockcroft, S. 1998).

The mammalian FYVE finger proteins EEA1, Hrs, and FGD1 (Faciogenital dysplasia gene product) are unrelated and have no similarity to SARA outside of the FYVE domain. This suggests that FYVE domains may possess a common functional role in various structurally distinct proteins. The FYVE domains of EEA1, and Hrs have been shown to bind directly to phosphatidyl inositol-3'-phosphate (PI(3)P) with high specificity (Gaullier, J.-M. et al. 1998; Patki, V. et al. 1998). Structural analysis of the FYVE domains of EEA1 (Kutateladze T.G. et al. 1999) and Vps27p (Misra, S. and Hurley, J.H. 1999) have shown that this interaction is mediated through two antiparallel beta sheets and an alpha helix stabilized by two Zn2+ binding clusters. Sequence comparison of the FYVE domains of Hrs, EEA1 and SARA reveals a high degree of conservation of residues, suggesting that the FYVE domain of SARA may also bind PI(3)P and function to bring SARA to the membrane.
Figure 1

(A) Sequence Alignment of FYVE domains

An alignment of the amino acid sequences of the FYVE domains of human SARA (aa 587-655), Xenopus SARA (aa 510-578), Drosophila SARA (aa 521-589), and Hgs (aa 153-219) proteins. Conserved amino acids are highlighted in dark grey. A consensus sequence, in which all amino acids are identical, was derived from this alignment.

(B) Structure of hSARA

The FYVE domain (aa 587-655) and the Smad Binding Domain (SBD) (aa 665-706) of hSARA are shown in solid black and stripes, respectively. The corresponding amino acids are indicated above the schematic.
1.2.2.2 The Smad Binding Domain (SBD)

Tsukazaki and colleagues demonstrated that SARA interacts directly and specifically with the Smad2 MH2 domain (Tsukazaki, T. et al. 1998). A series of SARA deletion mutant constructs were used to narrow down the Smad2 binding region. A region that was necessary and sufficient for Smad2 interaction was mapped to 85 amino acids (aa 665-750), and termed the Smad Binding Domain (SBD). Recently, it was demonstrated that at least four residues in SARA, Tyr 680, Cys 681, Pro 686, and Val 703, play essential roles in Smad2 binding (Wu, G. et al. 2000). Moreover, the crystal structure of the SBD of SARA in complex with a Smad2 MH2 domain has been resolved to a resolution of 2.2Å (Wu, G. et al. 2000). In these crystal structures, SARA SBD binds to Smad2 via an extended region in the SBD that includes a rigid coil, an alpha-helix, and a β-strand. A schematic diagram of the structure of hSARA indicating its domains is shown in Figure 1B.

1.2.3 The role of SARA in TGFβ signaling

Initial characterization of mammalian SARA has defined it as an important component of the TGFβ signaling pathway (Tsukazaki, T. et al. 1998). In particular, it has been shown that SARA interacts specifically with Smads 2 and 3, the downstream mediators of TGFβ signaling. In addition, confocal immunofluorescence microscopy has revealed that SARA recruits Smad2 to specific subcellular sites of action to which the TGFβ receptors localize. Furthermore, association of SARA with the TGFβ receptor complex was demonstrated. Deletion mutant analysis defined the FYVE domain as necessary for the correct subcellular localization of SARA. Remarkably, SARA mutants (ΔFYVE) that mislocalize Smad2 block TGFβ signaling. Based on these findings, it has
been proposed that SARA recruits Smad2 to the TGFβ receptor complex by controlling its subcellular localization.

1.3 Current Model of TGFβ Signal Transduction

The current model for TGFβ signal transduction may be summarized as follows. Activation of the pathway occurs when TGFβ ligand binds the type II receptor, which results in the recruitment of the type I receptor into a heteromeric receptor complex. The constitutively activated type II receptor kinase then transphosphorylates and activates the type I receptor. Subsequently, SARA recruits Smad2 to this activated receptor complex, where it is phosphorylated by its type I receptor. Upon phosphorylation, Smad2 dissociates from this complex, oligomerizes with Smad4, and translocates to the nucleus where it regulates downstream target gene expression. This model is depicted in Figure 2.

1.4 Thesis Rationale, Objectives and Hypothesis

Numerous studies in the TGFβ field have advanced our understanding of this signaling pathway, however, little is known about the regulation of Smad subcellular localization. Although the initial characterization of SARA has provided some insight into this process, the role of SARA in TGFβ signaling has not been fully elucidated. Thus, it is the overall goal of my study to further characterize SARA and its function in this pathway. In particular, my first objective was to analyze the expression pattern of SARA with respect to Smad2, and to identify the subcellular vesicles to which SARA localizes. Other goals included the investigation of the specificity of SARA in TGFβ superfamily signaling. My final aim involved further characterization of the association of SARA and the TGFβ receptors. I hypothesize that SARA is a scaffold that anchors the type I receptor in close proximity to its substrate, Smad2.
Figure 2. A Model for the initiation of the TGFβ signaling pathway.

In this model, the ligand associated type II receptor transphosphorylates and activates the type I receptor. SARA interacts with PI(3)P through its FYVE domain, and anchors Smad2/3 through interactions with the SBD. SARA, recruits Smad2/3 to this activated receptor complex, which allows the type I receptor to phosphorylate Smad2/3. Upon phosphorylation, Smad2 dissociates from the receptor complex, binds to Smad4, and translocates to the nucleus.
MATERIALS AND METHODS

2.1 Cell Culture

HepG2 and MvlLu cells were maintained in minimal essential media (MEM) with 10% fetal bovine serum (FBS) and nonessential amino acids (Gibco-BRL). COS-1 and 293T cells were cultured in Dulbecco's Modified Eagle Media (DMEM) and 10% FBS (Gibco-BRL). All cell lines were cultured at 37°C in a 5% CO₂ environment.

2.2 RT-PCR Analysis

RT-PCR analysis was performed to assess the expression of SARA RNA in various cell lines. Total RNA was isolated from HepG2 hepatoma cells, NBFL neuroblastoma cells, SW480 colorectal cancer cells, NIH 3T3 fibroblasts, and MvlLu lung epithelial cells using the TRIzol™ reagent according to the manufacturer's recommendations (Gibco BRL). Reverse transcription was carried out using 10 μg of total RNA, 2.5 μM random hexamer primers and 1 U/reaction of reverse transcriptase in a total volume of 20 μL. The reaction mix consisted of 5 mM MgCl₂, 1XPCR buffer, diethylpyrocarbonate (DEPC) dH₂O, 1 mM of each dNTP (Pharmacia Biotech) and 1 U/μL RNase inhibitor (Promega). All reagents were obtained from Perkin Elmer unless otherwise stated. A negative control (-RT), which included all of the components of the mix except the enzyme, was set up for each type of RNA. Following a 10 minute room temperature incubation, this reaction mix was incubated at 42°C for 45 minutes, 99°C for 5 minutes and then at 4°C.

The reverse transcribed RNA samples from the human cell lines (HepG2, NBFL, and SW480) were amplified using the following primers that were derived from the hSARA nucleotide sequence:
Forward: 5'...GCCGTCGACCATCTTTGGGAAACATCTCTA...3'
Reverse: 5'...AACTCGCCTCTGCTCTCT...3'

Each PCR cycle (total of 30) was carried out as follows: a denaturation step at 95°C for 15 seconds (sec), an annealing step at 55°C for 30 sec, and an extension phase at 72°C for 30 sec, followed by a final 10 minute extension at 72°C. The expected size of the resulting PCR product was 575 bp. The PCR reaction mixes consisted of: 5 μL of the RT reaction, 2 mM MgCl₂, 1X PCR Buffer II, 250 μM dNTP's (Pharmacia Biotech), 2.5 U/μL AmpliTaq DNA polymerase, dH₂O and 0.5 μM of each primer (total volume 50 μL). All components were purchased from Perkin Elmer unless otherwise indicated. PCR amplifications were carried out in a Perkin Elmer Gene Amplification PCR system 9600.

Degenerate primers were used to carry out PCR with cDNA transcribed from the non-human cell line (MviLu and NIH3T3) RNA. Based on the XSARA amino acid sequence, the primers were designed to encode the FYVE domain and have been previously used to clone hSARA (Tsukazaki, T. et al. 1998). The primer sequences are:

Forward: 5'...GC(a/c/g/t)CC(a/c/g/t)AA(c/t)TG(c/t)ATG(c/t)TG(c/t)...3'
Reverse: 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 resulting PCR reactions were subjected to 3 cycles of amplification at the annealing temperature of 42°C, followed by 40 cycles of amplification at an annealing temperature of 55°C as described in (Attisano, L. et al. 1992). The expected PCR product size was 250 bp. The same PCR conditions as described above were used. A 10 μL aliquot of the reaction was loaded onto a 1% TAE-agarose gel and positive amplification was determined by agarose gel electrophoresis with visualization by ethidium bromide staining.

2.3 Northern Blot Analysis

A human multiple tissue northern blot (Clontech), which had 2 μg of polyA⁺ RNA loaded per lane, was prehybridized in 10 mL of ExpressHyb solution (Clontech) at 68°C
for 30 minutes. cDNA probes were labelled with \(^{32}\text{P}\)-dCTP using the Ready To Go DNA Labelling Kit (Amersham) and subsequently purified in a G-50 Probe Quant column (Amersham). The blot was first hybridized to a 472 bp \textit{Apal/Smal} restriction fragment from the 3'UTR of \textit{hSARA} cDNA and subsequently to a 1.2 kb \textit{SalI/BglII Smad2} MH1 cDNA fragment. Hybridization was carried out in 10 mL of ExpressHyb solution at 68°C for 1 hour with ~2.0 x 10\(^6\) cpm/mL of \(^{32}\text{P}\)-labelled probes. The blot was washed at room temperature twice for 15 minutes with 2XSSC, 0.05 %SDS, and then twice for 40 minutes at 50°C with 0.1XSSC, 0.1% SDS. The membrane was then exposed to XAR film (Kodak) at -70°C with an intensifying screen. The blot was deprobed by two incubations, 5 minutes each, in freshly boiled 0.5% SDS.

### 2.4 Immunofluorescence and Confocal Microscopy

\textit{Mv1Lu} cells were plated onto gelatin-coated Permanox chamber glass slides (Nalgene Nunc). When the cells reached approximately 20% confluency, they were transiently transfected with the appropriate DNA constructs using the calcium phosphate (CaP\(_{0.4}\))-DNA precipitation method. Approximately 16 hours after DNA-CaP\(_{0.4}\) precipitate addition, the cells were washed with fresh media (MEM + NEAA + 10% FBS) and were incubated for another 24 hours before fixing and staining. The cells were washed with phosphate buffered saline (PBS), fixed with fresh 4% paraformaldehyde for 10 minutes at room temperature, washed with PBS and subsequently permeabilized in 100% methanol for 2 minutes at room temperature. After washing with PBS several times, the cells were then incubated with PBS containing 10% heat treated goat serum (Gibco BRL) for 1 hour at room temperature to block non-specific staining. Following blocking, the cells were incubated with the appropriate primary antibody in PBS containing 10% goat serum. For co-staining of Myc-SARA and EEA1, the anti-Myc-A14 rabbit polyclonal antibody (Santa Cruz) at 1 \(\mu\)g/mL was used to detect transfected Myc-hSARA, while the anti-EEA1 mouse
monoclonal antibody (Transduction Laboratories) at 1 µg/mL was used to detect endogenous EEA1. For co-staining of Flag-rab9 and Myc-hSARA, anti-Flag-M2 monoclonal antibody (Sigma) at 10 µg/mL was used to detect Flag-rab9 and anti-Myc A14 polyclonal antibody was used to detect Myc-hSARA. For co-staining with Flag-SARA, Hgs tagged with the hemagglutinin HA epitope was detected with anti-HA Y-11 rabbit polyclonal antibody (Santa Cruz) at 1 µg/mL. The unbound primary antibody was removed in 5 washes with PBS over a 5 minute period. The appropriate fluorescent conjugated secondary antibodies were added to the cells for 1 hour in the dark at room temperature. FITC-conjugated goat anti-mouse antibodies were used to detect the anti-Flag-M2, and anti-EEA1 monoclonal antibodies, while Rhodamine-Texas Red-conjugated goat anti-rabbit antibody was used to detect the anti-HA and anti-Myc polyclonal antibodies. These fluorescent conjugated antibodies (Jackson Laboratories) were used at a 1:200 dilution in 10% goat serum. The cells were washed again 5 times with PBS over a 5 minute period, and then stained for nuclei using 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma) at a final concentration of 1 µg/mL in PBS for 3 minutes. Mowiol and DABCO (Mowiol 4-88 (Hoechst), glycerol, Tris-HCl, 1,4-diazobicyclo-[2.2.2]-octane (DABCO)) (Harlow, E. and Lane, D. 1998) were then added to the cells. Confocal analysis was performed using a Leica Confocal microscope on the stained cells.

2.5 Subcloning

In order to narrow down the region in SARA important for receptor interaction, a series of SARA deletion mutants were constructed. The generation of these constructs was accomplished using common subcloning strategies, such as restriction digestions, PCR, Klenow polymerase fill ins, and ligations. All SARA constructs were subcloned into the pCMV5 mammalian expression vector containing an amino terminal Flag epitope (Hoodless, P.A. et al. 1996). All restriction sites were introduced into Flag-SARA using
PCR with the appropriate primers. Flag-SARA has a SalI site immediately following the Flag epitope preceding the SARA coding sequence, a unique internal EcoRV site, and a Smal site at the end of the SARA coding sequence (aa 1323) that is followed by stop codons. SARA mutants aaΔ1-752 and aaΔ1-803 were constructed by introducing a SalI site via PCR, at amino acids 752 and 803 respectively. These PCR products were restriction digested with SalI and EcoRV and subsequently subcloned into SalI/EcoRV wild-type Flag-SARA. To generate the aaΔ1-1000 construct, SalI and Smal sites were introduced at amino acids 1000 and 1323, respectively. The resulting PCR product was digested with SalI and Smal and subcloned into SalI/Smal Flag-SARA. The aaΔ797-1323 construct was generated by introducing a Smal restriction site at amino acid 796. The resulting PCR product was digested with XbaI and Smal and subsequently subcloned into Flag-SARA (XbaI/Smal). To make aaΔ1016-1323, Flag-SARA was AccI digested, Klenow filled in with nucleotides to form a blunt end, and subsequently XbaI digested. This product was then subcloned into Flag-SARA, which had been HindIII digested, Klenow filled in with nucleotides, and XbaI digested. The correct sequence and reading frame were verified by DNA sequencing. Their expression was confirmed by Western blot analysis using the anti-Flag antibody since all constructs have an amino-terminal Flag tag. Only constructs that expressed efficiently were used in the deletion mutant analysis.

2.6 Production and Purification of Glutathione-S-Transferase Fusion Proteins

In order to construct GST-FYVE domain fusion proteins, DNA encoding the SARA FYVE domain was subcloned into the pGEX-4T1 vector. In particular, EcoRI and SalI sites were introduced into the SARA cDNA sequence via PCR at base pairs 1588 and 2002, respectively. The resultant PCR product was digested with EcoRI and SalI, and was subcloned into pGEX-4T1 (EcoRI/SalI). DH5α E.coli cells were transformed with the pGEX-4T1 fusion constructs and plasmid DNA purified from single colonies of these cells
was sequenced to verify that the constructs were correct. Transformants were inoculated in 5 mL of LB containing ampicillin (100 µg/mL) and were grown overnight shaking at 37°C. The following day, 1 mL of culture was diluted into 10 mL of LB containing ampicillin and grown at 37°C until reaching an approximate OD (optical density) of 0.7. To induce protein expression, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture for 3 hours. The bacteria was spun down for 10 min at 5000 x g. The supernatant was discarded and the remaining pellet was lysed with 1 mL of TNTE (0.5% Triton X-100) buffer, which consisted of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 10 µg/mL Pepstatin A (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µg/mL soybean trypsin inhibitor (Roche), 50 µg/mL leupeptin (Roche), 10 µg/mL antipain (Sigma), and 50 µg/mL apronitin (Roche). The cells were transferred to eppendorf microcentrifuge tubes and sonicated (Vibrasonic sonicator) on ice for 15 sec 3 times. The cells were then microcentrifuged for 5 min at 4°C. The supernatants were collected and subsequent purification of the GST-fusion proteins was carried out using Glutathione Sepharose 4B (Pharmacia Biotech) according to the manufacturer’s instructions. The eluted fusion proteins were then resolved by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie staining.

2.7 Transient Transfection of DNA in Mammalian Cells

All co-immunoprecipitation studies were done with COS-1 simian cells or 293T human cells that were transiently transfected using the LipofectAMINE™ reagent (Gibco/BRL). In a six-well dish, ~2x10⁵ cells per well were seeded in 2 mL DMEM. These cells were grown at 37°C in a CO₂ incubator until reaching 50-80% confluency (approximately 18-24 hours). Transfection was performed as directed in the procedure described by Gibco/BRL, where 2.5 µg total DNA was used per well.
2.8 Cell Lysate Preparation and Immunoprecipitation

Cells were washed with ice cold 1X phosphate buffered saline (PBS). They were subsequently lysed with TNTE (0.5% Triton X-100) buffer which consisted of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 10 µg/mL Pepstatin A (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 µg/mL soybean trypsin inhibitor (Roche), 50 µg/mL leupeptin (Roche), 10 µg/mL antipain (Sigma), 50 µg/mL aprotinin (Roche), and 1mM Na3VO4. Lysis was performed for 15 min at 4°C. The lysates were collected and centrifuged at 4°C for 10 min. A fraction (70 µL) of the cleared lysate was added to 8X SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) sample buffer to be used for Western blot analysis to determine protein expression. The remaining lysate was subjected to immunoprecipitation. The 8X sample buffer consisted of 0.4 M Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 0.5% bromophenol blue and 0.4 M DTT.

The anti-Flag M2 antibody (Sigma) at a 1:1000 dilution and the monoclonal 12CA5 anti-HA antibody (mouse hybridoma cell line supernatant) at a 1:50 dilution were used to immunoprecipitate Flag-tagged and HA-tagged proteins, respectively. After incubation with the primary antibody for 1 hour at room temperature, protein G sepharose (Pharmacia) was added to the lysates and rocked gently at 4°C for 1 hour. The beads were then washed 5X with 750 µL of TNTE (0.1% Triton-X). After 50 µL of 2X sample buffer was added to the washed beads, the proteins were heated to 100°C for 3 minutes. Type I TGFβ receptors migrate to the same position as the reduced heavy chain of the anti-Flag antibody and are therefore difficult to detect as co-immunoprecipitating proteins. Therefore, when the co-immunoprecipitating protein to be analyzed was a type I receptor, an M2 anti-Flag affinity gel (Sigma) was used to immunoprecipitate the Flag-tagged protein. This affinity gel consists of the M2 anti-Flag antibody bound to agarose beads. The Flag-protein can be
eluted off the gel with 2X sample buffer (without DTT) without co-elution of the antibody heavy chain.

2.9 SDS-PAGE and Western Blotting

Immunoprecipitates or protein from cell lysates were resolved on 8% separating and 3.5% stacking SDS-polyacrylamide gels. The marker SeeBlue (Novex) was run in each gel. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Xymotech). The transfers were performed at 4°C for 1 hour at 100 V in transfer buffer which consisted of 25 mM Tris, 192 mM glycine and 20% methanol. After the transfer was complete, the membranes were stained with Ponceau S (Sigma) to verify the transfer of the protein. The blot was then washed with TBST (200 mM Tris-HCl, 0.5 M NaCl, pH 7.2 and 0.1 % Tween-20). The blots were blocked for 1 hour at room temperature with 5% skim powder milk in TBST to prevent non-specific protein binding. The blots were then incubated at room temperature for 1 hour with the appropriate primary antibody diluted in TBST. The anti-Flag M2 monoclonal antibody (Sigma), an anti-Hgs polyclonal antibody (generously provided by Dr. Sugamura), the anti-HA Y-11 rabbit polyclonal antibody (Santa Cruz), and the anti-Myc A14 polyclonal antibody (Santa Cruz) were used to detect Flag-tagged, endogenous Hgs, HA-tagged and Myc-tagged proteins, respectively. All antibodies were used at a 1:1000 dilution in TBST except the anti-Flag antibody, which was used at a 1:5000 dilution. To remove unbound primary antibody, the blots were washed 5X 5 min in TBST. Subsequently, the blots were incubated with a horseradish peroxidase conjugated goat anti-rabbit IgG or goat anti-mouse secondary antibody diluted 1:10000 in TBST for 1 hour at room temperature. The membranes were washed again as described before. The blots were mixed in a solution containing 3 mL of each of the two enhanced chemiluminescence reagents (Amersham) for 1 min and exposed to XAR film (Kodak) to visualize the protein.
RESULTS

3.1 Expression analysis of SARA

To investigate the expression pattern of SARA, the presence of SARA RNA was tested in various human and non-human cell lines. Secondly, the level of expression of SARA in comparison to Smad2 was examined in multiple human tissues.

3.1.1 Examination of SARA expression in various cell lines using RT-PCR

In order to assess the expression of SARA, total RNA was isolated from human cell lines including HepG2, a human hepatoma cell line, NBFL, a neuroblastoma cell line, and SW480, a colorectal cell line. In addition, total RNA was isolated from non-human cell lines including NIH3T3, a mouse fibroblast cell line, and Mv1Lu mink epithelial cells. cDNA was reverse transcribed from this RNA and then PCR amplified. Primers specific to the hSARA cDNA sequence were used to carry out PCR for the human cell derived cDNA, while degenerate primers that were based on the XSARA amino acid sequence flanking the FYVE domain and SBD, were used for the non-human cell derived cDNA. A PCR product of the expected size of 575 bp, was observed in all three human cell lines tested (Figure 3A). A PCR product of the expected size of 250 bp, was observed in the non-human cell lines (Figure 3B). Thus, SARA is expressed in multiple cell lines, which is consistent with the plethora of roles of TGFB signalling (Roberts, A.B. and Sporn, M.B. 1990).
Figure 3. SARA expression analysis in multiple cell lines using RT-PCR

(A) Total RNA was isolated from human cell lines including HepG2 hepatoma cells, NBFL neuroblastoma cells, and SW480 colorectal cancer cells. cDNA, which was reverse transcribed from this RNA, was PCR amplified using primers specific to the human SARA cDNA. Reverse transcription was carried out in the absence (-RT) or presence (+RT) of reverse transcriptase. A PCR product of the expected size of 575 bp, is observed in each tested cell line.

(B) The expression of SARA was tested in the non-human cell lines NIH3T3 fibroblasts and MvILu lung epithelial cells. cDNA was reverse transcribed from total RNA that was isolated from these cell lines in the presence (+RT) or absence (-RT) of reverse transcriptase. PCR amplification of this cDNA was carried out using degenerate primers. A 250 bp PCR product (expected size) is observed in each cell line tested.
Figure 3
3.1.2 Northern Blot Analysis

In order to investigate the expression pattern of SARA with respect to that of Smad2, an adult human multiple tissue northern blot (Clontech) was sequentially hybridized with radiolabeled hSARA and Smad2 cDNAs. The 3' untranslated region (UTR) of SARA was used as a probe because, in contrast to coding sequence, it is unlikely to be conserved in potential SARA homologues, thus ensuring that detected transcripts would correspond to our SARA clone. A single transcript of 5.0 kb, which corresponds to the full length SARA cDNA, was observed in each tissue of the blot (indicated in upper panel, Figure 4). The 4.0 and 2.7 kb alternatively spliced forms of Smad2, which have previously been reported (Takenoshita, S. et al. 1998), were also expressed in all tissues (indicated in lower panel, Figure 4). SARA and Smad2 transcripts were co-expressed at high levels in heart, muscle, kidney and pancreas tissues, and at lower levels in lung and liver tissues. The transcript in the liver tissue was very faint, however, higher exposures demonstrated that SARA and Smad2 are expressed in this tissue (data not shown). Thus, SARA and Smad2 are both expressed in a similar pattern in multiple adult human tissues. Interestingly, this similar pattern of expression was not observed in all tissues. In particular, brain tissue appeared to express higher levels of SARA transcripts relative to Smad2, while placental tissue expressed Smad2 at higher levels than SARA. These results reveal that SARA and Smad2 are widely expressed in adult tissues, supporting the view that SARA is an important component of the TGFβ pathway.

3.2 Subcellular localization of SARA

SARA localizes to punctate subcellular microdomains to which it anchors Smad2. SARA mutants (Δ1-664), which interact with Smad2 but lack the FYVE domain, mislocalize
Figure 4. SARA and Smad2 expression patterns in different human tissues

An *Apal-Smal* restriction fragment from the 3'UTR of hSARA was used to probe a human multiple tissue northern blot (Clontech). Approximately 2 μg of polyA⁺ RNA of each tissue was loaded per lane. A single transcript of approximately 5.0 kb, which corresponds to the full length SARA cDNA, is indicated (upper panel). Subsequently, this blot was hybridized with a 1.2 kb *SalI/BglII* Smad2 MH1 cDNA fragment. The two alternatively spliced forms of Smad2, corresponding to 4.0 and 2.7 kb, are indicated (lower panel). SARA and Smad2 transcripts coexpress at high levels in heart, muscle, kidney and pancreas tissues. In contrast, these transcripts express at much lower levels in lung and liver tissues. Brain tissue has higher levels of transcript expression of SARA than Smad2, while placental tissue expresses Smad2 at higher levels than SARA.

(Note: This figure has been published under the title: SARA, a FYVE Domain Protein that Recruits Smad2 to the TGFβ Receptor. Tomoo Tsukazaki, Theodore A. Chiang, Anne F. Davison, Liliana Attisano, and Jeffrey L. Wrana. Cell 95 (6), 779-791. Dec. 11, 1998.)
Figure 4
Smad2 into the cytoplasm away from these subcellular regions. The overexpression of such mutants in HepG2 and Mv1Lu cell lines has been shown to block TGFβ dependent transcriptional responses (Tsukazaki, T. et al. 1998). This demonstrates that the correct localization of Smad2 is important in TGFβ signaling. Therefore, it was of interest to identify the punctate subcellular compartments to which SARA localizes. To accomplish this, colocalization studies with various subcellular marker proteins were carried out using confocal double label immunofluorescence microscopy. The punctate staining pattern of SARA is reminiscent of the localization patterns of endosomes, therefore, early and late endosomal markers were chosen for this study. In particular, localization of SARA with respect to early endosome autoantigen 1 (EEA1), an early endosome marker (Mu, F.T. et al. 1995), and rab9, a late endosome marker (Lombardi, D. et al. 1993), was analyzed (Figure 5). Mv1Lu cells were transiently transfected with Myc-tagged SARA (red), which was visualized by polyclonal rabbit anti-Myc antibody and Texas red-conjugated goat anti-rabbit antibody. Endogenous EEA1 (green) was visualized by mouse anti-EEA1 antibody and FITC-conjugated goat anti-mouse antibody. Extensive colocalization of SARA and endogenous EEA1 is seen as yellow in the overlay (Figure 5A). This suggests that SARA resides in early endosomes. Myc-tagged SARA and Flag-tagged rab9 were also co-expressed in Mv1Lu cells. It should be noted that overexpressed rab9 remains accurately targeted to late endosomes (Lombardi, D. et al. 1993). Myc-tagged SARA (red) was visualized by polyclonal rabbit anti-Myc and Texas red-conjugated anti-rabbit antibodies while Flag-rab9 (green) was visualized by mouse anti-Flag and FITC-conjugated anti-mouse antibodies. In contrast to the colocalization observed with Myc-SARA and EEA1, Myc-SARA and Flag-rab9 do not appear to colocalize at all (note the absence of yellow in the overlay; Figure 5B). These results strongly suggest that SARA localizes specifically to early, but not late, endosomes.
Figure 5. SARA subcellular localization

The localization of SARA with respect to subcellular protein markers was analyzed using double label immunofluorescence and confocal microscopy analysis.

(A) The localization of SARA with respect to early endosomal marker EEA1. Mv1Lu cells were transiently transfected with Myc-SARA cDNA alone. Myc SARA (red) was visualized using the polyclonal A14 rabbit anti-Myc antibody and Texas red-conjugated goat anti-rabbit antibody. Endogenous EEA1 (green), an early endosomal marker, was visualized using the mouse anti-EEA1 antibody and the FITC conjugated goat anti-rabbit secondary antibody. Extensive colocalization of Myc-SARA and endogenous EEA1 is observed as yellow in the overlay.

(B) The localization of SARA with respect to late endosomal marker rab9. Mv1Lu cells were cotransfected with Myc-SARA and Flag-rab9 cDNA. The cells were incubated with rabbit anti-Myc and mouse anti-Flag antibodies and subsequently with Texas red conjugated anti-rabbit and FITC-conjugated anti-mouse secondary antibodies. In contrast to SARA and EEA1, Myc-SARA (red) and Flag-rab9 (green) do not appear to colocalize (as observed by the absence of yellow in the overlay).
Figure 5
The only known phosphoinositide 3'-kinase (PI(3)K) in yeast is Vps34p, which catalyzes the conversion of phosphatidyl inositol (PI) to PI(3)P (Schu, P.V. et al, 1993). In contrast, several mammalian PI(3)K isoforms have been identified, which are classified into three groups based on their substrate specificity (Waterfield et al, 1997). In particular, PI, PI(4)P and PI(4,5)P2 are substrates for the class I PI(3)K isoforms, PI and PI(4)P are substrates for class II isoforms, whereas PI is the only substrate of class III PI(3)K's (Vanhaesebroeck, B. et al, 1997). The predominant products of the class I PI(3)K's are PI(3,4)P2 and PI(3,4,5)P3, and the major producer of PI(3)P in mammalian cells is the class III PI(3)K (Auger, K.R. et al. 1989; Patki, V., et al. 1997). Unlike the other PI(3)K isoforms, class III PI(3)K's are not regulated by growth factors and are thought to constitutively produce PI(3)P that is required for membrane trafficking.

The FYVE domains of EEA1, Hrs/Hgs and Vps27p interact directly and specifically with PI(3)P (Burd, C. and Emr, S. 1998; Gaullier, J.-M. et al. 1998; Patki, V. et al. 1998; Simonsen, A. et al. 1998; Wiedemann, C. and Cockcroft, S. 1998; Kutateladze T.G. et al. 1999; Misra, S. and Hurley, J.H. 1999). The FYVE domain of SARA, which is required for its proper subcellular localization, is highly conserved with the FYVE domains of EEA1, and Hrs/Hgs (Tsukazaki, T. et al. 1998). It is therefore possible that SARA also binds PI(3)P through its FYVE domain, and may be regulated by PI(3)K. To investigate whether the subcellular localization of SARA is dependent on PI(3)P, the effect of wortmannin on SARA localization was analyzed. Subsequently, fusion proteins of GST and amino acid regions containing the FYVE domains of EEA1 (aa 1307-1411; generously provided by Dr. H. Stenmark), and SARA (aa 530-668; Figure 6B) were used to analyze potential SARA FYVE domain interactions with lipids. To this end, in vitro liposome binding assays with these fusion proteins were performed (in collaboration with Dr. Silvia Corvera).
Figure 6

(A) Immunofluorescence confocal microscopy analysis was used to observe the effect of wortmannin, a PI(3)K inhibitor, on the subcellular localization of SARA. MvILu cells that were transiently transfected with Myc-SARA cDNA were treated in the presence (ii) or absence (i) of wortmannin (100 nM). Myc-SARA (red) was visualized with A14 rabbit anti-Myc antibody and Texas red-conjugated goat anti-rabbit antibody. Untreated cells displayed the characteristic punctate staining pattern of SARA (i), while the majority of SARA exhibited a diffuse staining pattern in wortmannin treated cells (ii).

(B) Production and purification of GST-FYVE fusion proteins. GST-FYVE fusion proteins of the SARA (aa 530-668) and EEA1 (aa 1307-1411) FYVE domains were generated and purified on glutathione-sepharose beads. Purified protein that was 10-fold and 100-fold diluted was resolved by SDS-PAGE and visualized by Coomassie staining. The sizes (kDa) of the low molecular weight (LMW) marker loaded in the first lane are indicated on the left. A GST control was loaded in the second and third lanes. The GST-FYVE domains of SARA and EEA1 are approximately 42 kDa as indicated by arrows. These proteins were subsequently used for in vitro liposome binding assays.
The fungal metabolite, wortmannin, irreversibly inhibits mammalian PI(3)K activity at a 50% inhibitory concentration (IC$_{50}$) of 5 nM (Zvelebil, M.J. et al. 1996; Cross, M. et al. 1995; Ui, M. et al. 1995), but does not inhibit the structurally related yeast Vps34p PI(3)K (Stack, J.H. et al., 1994). In addition, wortmannin has been shown to inhibit phospholipase A$_2$ activity at an IC$_{50}$ of less than 5 nM (Cross, M.J. et al., 1995), and some PI(4)K isoforms and myosin light chain kinase at an IC$_{50}$ greater than 200 nM (Meyers, R. et al., 1997). Thus, phosphatidylinositol (PI) would not be converted to PI(3)P in the presence of 100 nM wortmannin, and therefore, this metabolite was used to assess the effect of the absence of PI(3)P on SARA subcellular localization.

Mv1Lu cells were transiently transfected with Myc-SARA in the absence or presence of wortmannin (100 nM). Myc-SARA was visualized by confocal immunofluorescence microscopy of cells that were fixed and incubated with A14 rabbit anti-Myc antibodies and Texas red-conjugated anti-rabbit secondary antibodies. In the absence of wortmannin, SARA exhibited its characteristic punctate staining pattern (Figure 6A(i)). In contrast, this localization pattern was disrupted to a severe degree in the presence of wortmannin and SARA displayed diffuse staining, however, a visible portion of SARA remained punctate (Figure 6A(ii)). These results suggest that PI(3)P may play a role in promoting the correct localization of SARA. Consistent with these results, it has been observed that wortmannin disrupts the subcellular localization of the majority, but not all of EEA1 (Dr. Silvia Corvera, personal communication). EEA1 binds PI(3)P through its FYVE domain, and rab5 via a domain immediately amino-terminal of its FYVE finger (Simonsen, A., et al., 1998). Perhaps, similar to EEA1, SARA binds an endosomal protein in cooperation with the FYVE domain-PI(3)P interaction to ensure its proper localization.

To determine whether SARA-FYVE and PI(3)P directly interact, GST-FYVE SARA (aa 530-667) fusion proteins were generated (Figure 6B). Liposomes composed of phosphatidylserine and phosphatidylinositol (PS/PI) and different forms of phosphorylated
PI such as PI(3)P, PI(3,4)P₂, PI(3,4,5)P₃, PI(4)P, and PI(4,5)P₂ were incubated with GST-FYVEₛₐₐₕₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐCentury 00 - 1900 years

3.4 Characterization of interaction between SARA and the TGFβ superfamily of receptors

Tsukazaki and colleagues have recently shown that SARA associates with the TGFβ receptor complex (Tsukazaki, T. et al. 1998). In these studies, COS-1 cells were transfected with Flag-SARA and the type I and type II TGFβ receptors, and then incubated with [125I]-TGFβ to induce the formation of a radioactive signaling receptor complex. Lysis of these cells, followed by SARA immunoprecipitation resulted in the co-precipitation of radioactive receptor complexes, which were detected by SDS-PAGE and autoradiography. These results demonstrated that SARA interacts, either directly or indirectly, with the TGFβ receptor complex. In this experiment, the type I and type II receptors were cross-linked so it was unknown whether SARA associates with the type I, the type II receptor, or both receptors. In order to characterize the interaction between SARA and the receptors, I undertook a biochemical analysis to investigate the binding specificity and signaling dependence. In addition, I further mapped the region of SARA required for this association.
3.4.1 Specificity of interaction between SARA and the TGFβ receptors

To gain insight as to whether SARA acts exclusively in TGFβ signaling or whether it plays a role in other pathways of the TGFβ superfamily, the specificity of interaction between SARA and the type I receptors was analyzed. The type I receptors were used because they activate Smads and determine the specificity of downstream signaling. To accomplish this, COS-1 cells were transiently transfected with cDNA of Flag-tagged SARA along with HA-tagged forms of the various type I receptors. These cell lysates were immunoprecipitated for SARA with anti-Flag antibody and co-precipitating receptors were detected by SDS-PAGE and anti-HA antibody immunoblotting. As indicated (upper panel, Figure 7A), the orphan receptor, ALK1, the activin type I receptor, ALK4, and the TGFβ type I receptor, TβRI, all co-precipitate with Flag-SARA. In contrast, the BMP type I receptors, ALK2 and ALK6, co-precipitate with Flag-SARA at undetectable or very weak levels, respectively. The BMP type I receptor, ALK3, also does not associate with Flag-SARA (data not shown). To confirm that equal amounts of Flag-SARA were immunoprecipitated, this blot was subsequently probed with anti-Flag antibody (middle panel, Figure 7A). In addition, total lysates were analyzed by SDS-PAGE and anti-HA antibody Western blotting to verify receptor expression (bottom panel, Figure 7A).

The association between SARA and the type II receptors was also investigated, using the same method as described above. These results demonstrated that weak or undetectable levels of actRIIB, TβRII or actRII co-precipitated with Flag-SARA (upper panel, Figure 7B). Thus, SARA predominantly associates with type I receptors, and this interaction is specific to ALK1, ALK4 and TβRI.
Figure 7. Specificity of interaction between SARA and TGFβ receptors.

(A) Interaction of SARA and the type I receptors. COS-1 cells were transiently transfected with Flag-SARA cDNA either alone or together with the indicated C-terminal HA-tagged type I receptor cDNA. Cell lysates were subjected to anti-Flag antibody immunoprecipitation. Co-precipitating receptors were detected by SDS-PAGE and anti-HA antibody Western blotting (top panel). Equivalent expression and immunoprecipitation of Flag-SARA was determined by immunoblotting with anti-Flag antibody (middle panel). Efficient expression of the receptors was confirmed by SDS-PAGE and anti-HA antibody immunoblotting (bottom panel).

(B) Interaction of SARA and the type II receptors. COS-1 cells were transiently transfected with Flag-SARA cDNA either alone or together with the indicated C-terminal HA-tagged type II receptor cDNAs. Co-precipitating receptors, which were detected as described in (A), are indicated (upper panel). Efficient expression of Flag-SARA and the type II receptors was confirmed by immunoblotting total cell lysates with anti-Flag and anti-HA antibodies, respectively (middle and lower panels).
Figure 7
3.4.2 Signaling dependence of interaction between SARA and the type I receptors

To further understand the nature of the association between SARA and the type I receptors, this interaction was examined with different forms of the type I receptor. To this end, the wild-type (WT), constitutively activated (T206D), and kinase deficient (K233R) forms of HA-tagged ALK4 were co-expressed with Flag-tagged SARA in COS-1 cells. Following anti-Flag antibody immunoprecipitation of cell lysates, immunoprecipitates were resolved by SDS-PAGE, and co-precipitating receptors were detected via Western blot analysis with anti-HA antibodies (upper panel, Figure 8). All forms of ALK4 tested remained associated with SARA. These results suggest that SARA may act as a scaffold, whereby it binds with both the type I receptor and its downstream Smad substrate.

3.4.3 Mapping of regions in SARA important in mediating association with the type I receptors

Since previous studies have shown that the carboxy terminus of SARA is important for receptor interaction (Tsukazaki, T. et al. 1998), I was interested to test if this region mediates SARA interaction with the type I receptors. For this, various amino-terminal and carboxy-terminal deletion mutants were co-expressed along with HA-tagged ALK4 in COS-1 cells. Cell lysates were immunoprecipitated with anti-Flag antibody and co-precipitating receptor was detected via Western blot analysis using anti-HA antibody (upper panel Figure 9A). Anti-Flag antibody immunoblotting confirmed equivalent expression and immunoprecipitation of Flag-SARA (middle panel, Figure 9A). These results demonstrated that ALK4 associates with the full length, the amino terminal deletion mutant Δ1-664, the FYVE domain deletion mutant Δ597-664, and a mutant with both the FYVE domain
**Figure 8. Signaling Dependence of SARA-ALK4 Interaction**

COS-1 cells were co-transfected with Flag-SARA and the wild-type (WT), constitutively activated (T206D), and kinase deficient (K-R) forms of C-terminal HA-tagged ALK4 cDNAs. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody. Coprecipitating receptors were detected by SDS-PAGE and anti-HA antibody Western blotting (top panel). Aliquots of total cell lysates were immunoblotted with anti-Flag and anti-HA antibodies to confirm efficient expression of Flag-SARA and ALK4-HA, respectively (middle and lower panels).
Figure 8
and the SBD disrupted (Δ597-706). In contrast, a loss of interaction with SARA was observed with the carboxy-terminal deletion mutants Δ750-1323 and Δ665-1323. These results indicated that the carboxy-terminus (amino acids 750-1323) of SARA is important for association with ALK4.

In order to narrow down this receptor binding region, a series of further deletion mutants were generated (as summarized in Figure 9C). The same method as described above was used to determine if ALK4 associates with these mutants. These results demonstrated that SARA mutants Δ1-704, Δ1-752, Δ1-803, Δ1-892 and Δ1-1000 remain associated with ALK4-HA (upper panel, Figure 9B), thus, suggesting that the carboxy region (aa 1000-1323) in SARA is sufficient for receptor interaction.

3.5 Summary of Results

My studies demonstrate that SARA is ubiquitously co-expressed with Smad2 in human tissues. In addition, SARA localizes specifically to early endosomes, and PI(3)P participates in promoting this subcellular localization. Furthermore, the interaction of SARA with the type I receptors is specific for ALK1, ALK4, and TBRI. Moreover, this association is independent of signaling and is mediated by the carboxy 323 amino acids of SARA. Together, these findings provide a model of SARA as a scaffold for Smad2 and the type I receptor in the early endosome.
Figure 9. Mapping of the type I receptor binding domain in SARA.

(A) COS-1 cells were transiently transfected with ALK4-HA and with the indicated Flag-SARA deletion mutant constructs. Cell lysates were immunoprecipitated with anti-Flag antibody. Western blotting with anti-HA antibody was used to detect co-precipitating receptors (top panel), and with anti-Flag antibody to confirm equivalent expression and immunoprecipitation of Flag-SARA (middle panel). Total cell lysates were analyzed by SDS-PAGE and anti-HA antibody immunoblotting to confirm equal expression of the receptors (bottom panel). The deletion mutants used are depicted in (C).

(B) Subsequent SARA deletion mutants were generated (diagrammed in (C)). Using the method described above, the association between these SARA mutants and ALK4 was analyzed. Co-precipitation of ALK4 is indicated (upper panel). Expression of the SARA mutants and receptors was verified (middle and lower panels, respectively).

(C) The series of SARA deletion mutants used to map the receptor interacting domain are shown. The FYVE domain and SBD are depicted in solid black and striped black, respectively. The region important for receptor association is coloured in grey.
Figure 9

<table>
<thead>
<tr>
<th>Flag-SARA:</th>
<th>Full length (1-1323)</th>
<th>Receptor Binding Domain</th>
<th>ALK4 association</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK4-HA:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ1-664</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ1-704</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ1-752</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ1-903</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ1-992</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ1-1000</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ750-1323</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Δ855-1323</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Δ897-664</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Δ897-706</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
DISCUSSION

The plethora of recent studies in the field of TGFβ has greatly increased our knowledge of the components and mechanisms of this pathway. However, the regulation of these signals is less well understood. For example, the details of how Smad localization is controlled remain unclear. Initial characterization of the recently cloned SARA, has provided insight into some of these questions. In order to further understand the role of SARA in TGFβ signaling I have undertaken the analysis of this Smad2 anchor. In particular, I have demonstrated that SARA and Smad2 are widely co-expressed in multiple adult human tissues. Furthermore, dual label immunofluorescence confocal microscopy analysis has revealed that SARA localizes to early, but not late endosomes, and that PI(3)P plays a role in this localization. I also have characterized the association between SARA and the receptors of the TGFβ superfamily. I demonstrated that SARA interacts with the type I receptors, that this interaction is specific with ALK1, ALK4 and TβRI, and that SARA-receptor binding is not dependent on activation of the pathway. In addition, I have mapped a region in the carboxy-terminus of SARA that is required for its association with ALK4. Taken together, these results suggest a model in which SARA acts as a scaffold for the type I receptor and its substrate, Smad2, in early endosomes.

4.1 Characterization of SARA-Receptor Association

4.1.1 Specificity of SARA-receptor interactions

Activin and TGFβ signaling is initiated when the ligand induces heteromeric complex formation between its type II and type I receptors. This results in the phosphorylation of Smad2 and/or Smad3 by the type I receptor, followed by hetero-oligomerization with Smad4 and nuclear translocation (Baker, J.C. and Harland, R. 1996;
Lagna, G. et al. 1996; Macias-Silva, M. et al. 1996; Nakao, A. et al. 1997; Zhang, Y. et al. 1997). An analogous pathway has been defined for Smad1 (Hoodless, P.A. et al. 1996; Lagna, G. et al. 1996; Kretzschmar, M. et al. 1997; Macias-Silva, M. et al. 1998), Smad5 (Macias-Silva, M. et al. 1998), and Smad8 (Chen, Y. et al. 1997a) in response to BMP signaling. To investigate the specificity of the association between SARA and the type I receptors, I have carried out co-immunoprecipitation studies using COS-1 cells transfected with Flag-SARA and the different HA-tagged type I receptors. These results demonstrated that SARA interacts specifically with the TGFβ type I receptor, TβRI, the activin type I receptor, ALK4, and the orphan receptor, ALK1, in the absence of overexpressed type II receptor. Since SARA functions as a Smad2 anchor that does not bind Smads 1 or 5, it was not expected to associate with the BMP receptors, which is consistent with our observation that SARA does not bind ALK2, ALK3 or ALK6. The association of SARA with both ALK4 and TβRI suggests that SARA is a common component of the Smad2/3 pathways of activin and TGFβ. Surprisingly, SARA also associates with the type I receptor, ALK1. The role of ALK1 in signaling remains unresolved. Studies have shown that overexpressed ALK1 binds TGFβ in the presence of TβRII, and activin A in the presence of actRII (Attisano, L. et al. 1993; ten Dijke, P. et al. 1994), however, neither of these complexes elicits known TGFβ signals. ALK1 chimeras consisting of an ALK1 extracellular domain and a TβRI cytoplasmic domain are activated by TGFβ (Lux, A. et al. 1998). However, ALK1 has also been shown to activate Smad1 (Macias-Silva, M. et al. 1998; Chen, Y.-G. and Massagué, J. 1999) and Smad5 (Macias-Silva, M. et al. 1998), the downstream targets of BMP receptors. Perhaps, SARA plays a role in mediating cross talk between ALK1 and the Smad2/3 pathway, however, the function of SARA binding to ALK1 is difficult to interpret until the role of ALK1 is further defined.

SARA interacts with type I receptors and has low affinity for type II receptors. The proportion of the total amount of type II receptor that co-precipitates with SARA, however, is quite small (Figure 7B). This weak interaction may reflect the fact that type II receptors
are structurally related to type I receptors and thus, have some affinity for SARA. Alternatively, SARA may be associating with the type II receptor indirectly through endogenous type I receptor. As a result, it is currently unclear whether SARA-type II receptor interactions are reflective of what occurs with endogenous type II receptors.

4.1.2 Mapping of the Receptor Binding Domain

Through analysis of SARA deletion mutants, it was observed that all of the SARA mutants containing the carboxy-terminal 323 amino acids (aa 1000-1323) associated with the activin type I receptor. In contrast, SARA mutants lacking this region did not associate with ALK4 (Figure 9). These results thus demonstrate that the 323 amino acid region (aa 1000-1323) of SARA is sufficient to mediate ALK4 receptor association. Further deletion mapping and site directed mutagenesis must be carried out to ultimately define the minimal region required for this association. DSARA has also been shown to associate with the TGFβ and activin receptors (Teresa Reguly, personal communication), and the corresponding region in DSARA is similar to hSARA and may represent an evolutionarily conserved receptor interaction domain. Interestingly, unlike hSARA, DSARA additionally binds BMP receptors ALK3 and ALK6 (Teresa Reguly, personal communication). It will be of interest to determine which regions of DSARA and hSARA give rise to their different specificities of receptor interaction.

4.1.3 SARA as a Scaffold

Scaffold and anchoring proteins contribute to the specificity of signal transduction by recruiting active signaling molecules and placing them in close proximity to their substrates. Typically, scaffold proteins have docking sites for modular signaling proteins, and a membrane targeting element that tethers the complex to a specific subcellular
structure. For example, the prototypical scaffold, Sterile 5 (Ste5), is important for tight control of the pheromone Mitogen-Activated Protein Kinase (MAPK) pathway in Saccharomyces cerevisiae. Multiple docking sites on Ste5 serve to assemble the multiple components of the MAPK pathway (Choi, K.Y. et al. 1994). Localization of Ste5 at the plasma membrane is essential for the transmission of pheromone signals to associated MAPK cascade enzymes (Mahanty, S.K. et al. 1999). Similarly, my studies suggest that SARA may function as a scaffold. Firstly, it has a FYVE domain that targets it to early endosomes. Secondly, it recruits Smad2 to this region and binds the TGFβ type I receptor kinase (Tsukazaki, T. et al. 1998). Thus, SARA brings together the intracellular mediator of TGFβ signals, Smad2, and its upstream activator, the type I receptor. A demonstration that SARA, Smad2 and the type I receptor all occur in the same complex will support this model of SARA as a scaffold.

4.2 Model of SARA at the Early Endosome

SARA acts as an anchor for Smad2 and recruits it to the TGFβ receptor (Tsukazaki, T. et al. 1998). I have demonstrated that SARA colocalizes specifically with early endosomes and binds constitutively with the type I receptor, ALK4. Taken together, these results lead to a model of SARA acting as a scaffold at the early endosomes (depicted in Figure 10). I propose that SARA is localized to the cytoplasmic surface of early endosomes, where it holds the type I receptor in close proximity to its substrate, Smad2. How does the type II receptor access this complex? EEA1, with which SARA colocalizes in our studies, also colocalizes with transferrin receptor and rab5 (Mu, F.T. et al. 1995). Transferrin receptor was found to be associated with early endosomes that recycle to the plasma membrane (Sheff, D.R. et al. 1999). Therefore, SARA may also cycle to the plasma membrane where the ligand accessible type II receptors reside. Ligand bound type II receptor will hetero-oligomerize with type I, and this receptor complex may internalize along
with SARA and Smad2. Upon hetero-oligomerization, the type II receptor may transphosphorylate and activate the type I receptor, which may phosphorylate and activate Smad2. This phosphorylation event would result in the dissociation of Smad2 from SARA, which would initiate the propagation of downstream signaling events. This model is depicted in Figure 10.

This model suggests that TGFβ receptors may signal from the early endosome. This is consistent with the temporal time course of Smad activation, which is only achieved 15-30 minutes after TGFβ stimulation (Hoodless, P.A. et al. 1996). This is similar to other receptor systems. For example, internalized epidermal growth factor receptor (EGFR) and insulin receptors continue to signal from the early endosome (Baass, P. et al. 1995; Vieira, A.V. et al. 1996; Di Guglielmo, G.M. et al. 1998). Furthermore, although little is known of how TGFβ receptors internalize, TGFβ remains bound to its receptors at a pH of 5-5.5, the pH of early endosomes (Massagué, J. and Kelly, B. 1986). Furthermore, recent genetic studies suggest that endocytosis participates in Dpp signaling in wing development (Gonzalez-Gaitan, M. and Jackle, H. 1999). This is of special interest because Dpp is the *Drosophila* orthologue of BMP2/4, which has an analogous signaling pathway to TGFβ.

4.3 Functional Significance of SARA

Signaling events are numerous and highly integrated within the cell, and thus, must be tightly regulated. SARA contributes to this regulation in TGFβ signaling. By recruiting the type I receptor kinase and Smad2 to the endosome, SARA organizes this part of the TGFβ pathway into a spatially distinct functional entity. As a scaffold, SARA ensures that Smad2 is poised and ready for signaling events. This is critical when signal inducers, such as receptor kinases, are present at low levels (Dyson, S. and Gurdon, J.B. 1998) or when receptors signal for short time periods. Importantly, compartmentalization may prevent deregulated cross talk by Smad2 inhibitors, such as the Ras activated MAPK kinase.
In this model, SARA scaffolds the type I receptor and Smad2/3 via its carboxy terminus (aa 1000-1323) and its SBD, respectively. SARA recruits the type I receptor and Smad2/3 to the ligand bound type II receptor, and subsequently, type II phosphorylates and activates type I. This receptor complex internalizes along with SARA and Smad2/3. Smad2/3 is phosphorylated and activated by the activated type I receptor, and subsequently dissociates from SARA, binds Smad4 and translocates to the nucleus.
cascade (Kretzschmar, M. et al. 1999). Finally, SARA anchoring of Smad2 at the endosome may prevent uncontrolled signaling events due to inappropriate nuclear translocation of Smad2 in the absence of signal. SARA, therefore, contributes to the specificity and efficient regulation of TGFβ signal transduction.

4.4 Future Directions

To better understand its role in TGFβ signaling, further characterization of SARA is required. Demonstration that SARA, Smad2 and the type I receptor exist in one complex will support the model of SARA as a scaffold. It will also be of interest to define the residues in SARA and the type I receptor that are required for mediating their association. Additionally, identification of novel SARA interacting proteins would provide insight into other potential roles of SARA in TGFβ and other pathways. Finally, the generation of anti-SARA antibodies would be useful to confirm that the interactions and subcellular localization described in this study are consistent with endogenous levels of SARA.

4.4.1 Further defining SARA-type I receptor association

Co-immunoprecipitation studies using SARA deletion mutants and receptor chimeras will be used to determine the minimal amino acid segments required for SARA-receptor association. Receptor chimeras of ALK4 and ALK2, which have been constructed and generously provided by Dr. Derynck, will be used (Feng, X.-H. and Derynck, R. 1997). These constructs are appropriate because SARA associates specifically with ALK4 and not ALK2. In addition, chimeric constructs of these closely related receptors are less likely than deletion mutants to disrupt the proper folding of the kinase domain. Subsequent site directed mutagenesis of SARA and the receptor will further pinpoint specific residues that
are important for this interaction. These results will provide a molecular basis for the observed specificity of SARA-receptor interactions.

Similar to other scaffold proteins, SARA contains modular domains. For example, the FYVE domain of SARA is found within different contexts of the otherwise structurally unrelated proteins EEA1 and Hgs. Moreover, the SARA FYVE domain maintains its function even when fused with GFP; for example, GFP-FYVE binds specifically to PI(3)P and localizes to early endosomes (Silvia Corvera, personal communication). In addition, the SBD represents a modular domain that is functionally conserved in hSARA and DSARA (Teresa Reguly, personal communication). It will be interesting to see whether SARA binds directly to type I receptors, and if so, whether this involves a conserved modular domain. Presently, it remains unknown whether SARA and the receptors interact directly or indirectly. This is difficult to demonstrate \textit{in vitro} because the TGFβ receptors are not properly processed in non-mammalian systems. However, identification of the SARA binding domain of the receptor would allow us to address this question as the cytoplasmic portion of the receptor may be produced in insect cells (Huse, M. \textit{et al.} 1999).

**4.4.2 Identification of novel SARA interacting proteins**

The identification of novel SARA interacting proteins would provide insight into other potential functions of SARA. SARA has a large amino terminus (aa 1-596) that has no identified function. In addition, the carboxy terminus region (aa 704-1016) contains highly conserved sequences among hSARA, XSARA and DSARA. SARA may act as a scaffold for other proteins, and these conserved regions of SARA, or portions within them, may serve as additional docking sites. Approaches to identify interacting proteins will include phage expression library screening and mass-spectroscopic analysis of endogenous proteins that co-precipitate with SARA.
An expression library screen, developed by Young and Davis (Young, R. and Davis, R. 1983), involves the construction of a GST-fusion bait protein. The GST moiety is used for bacterial production and purification, and the protein kinase A (PKA) phosphorylation site in the construct is used for the radiolabeling of the fusion protein. IPTG induces expression of protein from the phage cDNA library. Radiolabeled GST-fusion bait protein is incubated with nitrocellulose lifts of each library plate. Lifts are washed and exposed to film to detect positive interactions. Positive clones are picked, purified and isolated for sequencing and further analysis. The use of a fusion protein as bait allows one to search for proteins that interact with a specified domain of SARA. In addition, this method results in the immediate availability of the cloned gene encoding the interacting protein and may provide information regarding a specific domain involved in this interaction. However, this method also has certain intrinsic limitations. For example, posttranslational modifications necessary for efficient binding may not occur in bacteria, and proteins that do not fold correctly in E. coli or do not maintain their structure on nitrocellulose filter will not be detected. Binding conditions are arbitrarily set and do not reflect the native cell environment so artifactual interacting proteins may be found. Finally, one is limited by the library type chosen as only those proteins expressed in the library may be detected.

An alternative approach to the expression library screen is mass spectroscopy analysis of interacting proteins. Lysates of mammalian cells transfected with Flag-tagged SARA are immunoprecipitated with anti-Flag antibody and run out on SDS-PAGE. Co-precipitating endogenous proteins are detected by silver staining. Specific interacting proteins are cut out, trypsin digested, and the peptide fragments that are produced are analyzed by mass spectroscopy. Mass spectroscopy analysis using Q-TOF yields a spectrum of the masses of the peptide fragments and tandem mass spectroscopy reveals partial amino acid sequences. This ‘tryptic fingerprint’ may be used to search databases in order to identify the interacting protein or to de novo sequence the peptide fragment. Unlike
the expression library screen, this method is advantageous as the protein interactions are detected in mammalian cells with endogenous amounts of the identified interacting protein. In addition, interactions may be analyzed under varying conditions, such as in the presence of different ligands. However, this method also has its limitations. Proteins that express at very low levels may be very difficult to detect. This method has recently been used to identify Protein Phosphatase 1 (PP1), as a SARA interacting protein (John Di Guglielmo, personal communication), and its role in TGFβ signaling is currently under investigation.
APPENDIX 1:

Analysis of Involvement of Hrs/Hgs FYVE Domain Protein in the TGFβ Signaling Pathway

5.1 Introduction

5.1.1 The FYVE Domain Protein Hgs

Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs/Hgs) was originally identified as a tyrosine-phosphorylated protein in cells in response to several growth factors including hepatocyte growth factor (HGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (Komada, M. and Kitamura, N. 1995). Hrs is also tyrosine phosphorylated in cells stimulated with cytokines interleukin-2 (IL-2) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Asao, H. et al. 1997). The Human Gene Nomenclature Committee has recently given the "Hrs" gene the approval symbol of Hgs. The human Hgs homolog is a 110-kDa protein that contains an amino-terminal conserved FYVE domain, a coiled-coil domain, a proline rich region and a carboxy-terminal proline- and glutamine-rich region. Hgs, like SARA, localizes to early endosomes, however, this localization does not appear to be dependent on its FYVE domain (Komada, M. et al. 1997). Hgs and its rat homolog, Hrs-2, are also thought to play a role in vesicular transport via endosomes (Bean, A.J. et al. 1997; Komada, M. et al. 1997). Additionally, Hgs associates with the signal-transducing adaptor molecule (STAM), and has been implicated in the regulation of IL-2 and GM-CSF signaling through counteraction of STAM function (Asao, H. et al. 1997).

Mice carrying a null mutation of the hgs gene have been generated (Komada, M. and Soriano, P. 1999). Hgs homozygous mutant embryos (Hgs -/-) died at approximately embryonic day 11 (E11) due to a defect in ventral folding morphogenesis. Moreover,
significant apoptosis was observed in the definitive endoderm of mutant embryos at E8.5. Abnormal enlargement of early endosomes and mislocalization of Hgs was observed in Hgs mutant cells, and wild type wortmannin-treated cells, respectively, implicating Hgs in the PI(3)K pathway of membrane trafficking.

Very recently, mice expressing a mutant form of Hgs, in which two exons including the coiled-coil domain of hgs were removed, have been generated (Miura, S. et al. 1999). These Hgs-/- embryos were lethal by E10.5, and at E6.5, E7.5 and E8.5, they were much smaller than the corresponding wild type embryos. RT-PCR gene expression analysis revealed that the brachyury (T) gene was not expressed at E6.5. Since expression of Brachyury has been shown to be mediated by activin signaling (Gu, Z. et al. 1998), this implicated Hgs in the activin pathway. Luciferase assays with an activin responsive promoter transfected into wild type or Hgs-/- embryos demonstrated that luciferase activity was 10 fold lower in mutant embryos upon activin stimulation. Moreover, Smads 1, 2 and 3 (in the presence of a cross linking agent) were shown to associate with full length Hgs and not the Hgs coiled-coil domain deletion mutant (Hgs-dCC). Finally, transfection studies revealed that wild type Hgs, but not Hgs-dCC, increased the association between Smad2 and the activin receptor complex. Thus, it was proposed that Hgs, in cooperation with SARA, recruits Smad2 to the receptor complex and is required for activin signaling in the early mouse embryo (Miura, S. et al. 1999).

5.1.2 Rationale and Objectives

The recent studies described above (Miura, S. et al. 1999) suggest that Hgs may be involved in activin signaling. In order to investigate the putative role of Hgs in activin signaling, I carried out a preliminary biochemical analysis of Hgs. In particular, I investigated potential interactions of Hgs with different components of the TGFβ family, and additionally, I examined the localization of Hgs with respect to SARA.
5.2 Results

5.2.1 Analysis of subcellular localization of Hgs with respect to SARA

Based on the model that Hgs acts in concert with SARA in activin signaling (Miura, S. et al. 1999), it may be expected that Hgs and SARA would colocalize. To test this possibility, I carried out confocal dual immunofluorescence microscopy analysis. Specifically, Mv1Lu cells were co-transfected with HA-tagged Hgs and Flag-tagged SARA cDNAs and following fixing and permeabilization, the cells were incubated with the appropriate antibody (Figure 11B). HA-Hgs (red) was visualized with polyclonal rabbit anti-HA and Texas-Red-conjugated anti-rabbit secondary antibodies (Figure 11B (i) and (iv)). Flag-SARA (green) was visualized with mouse anti-Flag antibody and FITC-conjugated anti-mouse secondary antibody (Figure 11B (ii) and (v)). Colocalization of HA-Hgs and Flag-SARA was observed as yellow in the overlay (Figure 11B (iii) and (vi)). As a control, Flag-SARA and HA-Hgs cDNAs were transfected individually in Mv1Lu cells to confirm that their localization pattern was not affected by the presence of the other protein (Figure 11A). It should be noted that the majority of cells that overexpressed Hgs had abnormally large endosomes. However, the images chosen as figures in this study represent cells expressing relatively low levels of Hgs with apparently normal endosomes. Thus, these results demonstrate that Flag-SARA and HA-Hgs partially colocalize in Mv1Lu cells, which is consistent with the model that they co-operate to function in activin signaling.

5.2.2 In vivo interaction of Hgs and specific TGFβ superfamily type I receptors

To investigate potential interactions between Hgs and the TGFβ family of receptors, COS-1 cells were transiently transfected with the different HA-tagged type I and type II
Figure 11. HA-Hgs colocalizes with Flag-SARA

(A) Transient transfection (Tfxn) of Mv1Lu cells was performed with either HA-Hgs (i) or Flag-SARA (ii) cDNA. HA-Hgs (red) was visualized with rabbit polyclonal anti-HA and Texas Red conjugated anti-rabbit antibodies (i). Flag-SARA (green) was visualized with mouse anti-Flag and FITC-conjugated anti-mouse antibodies (ii).

(B) Mv1Lu cells were co-transfected with HA-Hgs and Flag-SARA together. Flag-SARA (green) and HA-Hgs (red) were visualized as described in (A). Two different cells are presented with different stains ((i), (ii) and (iii) represent one cell, while (iv), (v) and (vi) represent a second cell). Some colocalization of Flag-SARA and HA-Hgs is observed as yellow in the overlay (iii) and (vi).
Figure 11
receptor cDNA. Anti-HA immunoprecipitates were resolved by SDS-PAGE and co-precipitating endogenous Hgs was detected by anti-Hgs Western blot analysis (upper panel, Figure 12). The detected Hgs band of 110-kDa corresponds to the expected size of the full-length protein. Total cell lysates were analyzed by SDS-PAGE and Western blotting with anti-Hgs and anti-HA antibodies to detect the expression of endogenous Hgs and transfected receptor, respectively (lower panel, Figure 12). These results demonstrate that endogenous Hgs co-precipitates with ALK1, the BMP2/4 type I receptors, ALK3 and ALK6, and the TGFβ type I receptor, TβRI. In contrast, Hgs does not co-precipitate with the type II receptors, the BMP7 type I receptor, ALK2, or the activin type I receptor, ALK4 (upper panel, Figure 12). This lack of interaction with ALK4 was surprising in consideration of the proposed role of Hgs in activin signaling.

To test the cell line specificity of this interaction, this experiment was repeated in 293T cells, which are derived from a transformed human kidney epithelial cell line. Overexpressed HA-tagged type I receptors were immunoprecipitated and co-precipitating endogenous Hgs was detected via Western blotting with anti-Hgs antibody. Anti-HA antibody Western blotting was performed to confirm that the receptors were efficiently expressed and immunoprecipitated. A 110-kDa protein corresponding to full length endogenous Hgs was detected in 293 total cell lysates at levels comparable to those observed in COS-1 cells (data not shown). Unexpectedly, interaction between endogenous Hgs and the type I receptors was not observed (data not shown), despite efficient expression and immunoprecipitation of receptors. Subsequently, potential interactions of the Smads and Hgs were tested. Co-immunoprecipitation studies, using COS-1 cells that were
Figure 12. Hgs interacts with specific type I receptors

(A) COS-1 cells were transiently transfected with HA-tagged TGFβ type I and type II receptor cDNAs. Anti-HA antibody immunoprecipitates were resolved by SDS-PAGE and co-precipitating endogenous Hgs was detected by anti-Hgs antibody immunoblotting (upper panel). To confirm efficient expression of the receptors and Hgs, total cell lysates were analyzed by anti-HA and anti-Hgs antibody Western blotting, respectively.
cotransfected with HA-tagged Hgs and Flag-tagged Smads 1, 2, 3, 4, and 6, were carried out as described above. No interaction was observed with Hgs and any of the Smads (data not shown).

These results demonstrate that association between endogenous Hgs and transfected type I receptors ALK1, ALK3, ALK6 and TßRI, occurs specifically in COS-1 cells and not in 293T cells. Moreover, Hgs and the type II receptors do not co-precipitate in either cell line. Additionally, association between HA-Hgs and Flag-tagged Smads is not detected in COS-1 cells in the absence of cross linking agent.

5.3 Discussion

Evidence that Hgs acts with SARA to recruit Smad2 to the activin receptor complex is very weak and my results suggest otherwise. The observed absence of brachyury gene (T gene) expression in the hgs-/- embryos (Miura, S. et al. 1999) was not necessarily caused by defective activin signaling since factors other than activin regulate T gene expression (Brancko, V.L. et al. 1997). Moreover, the goosecoid gene, which is a primary response gene induced by activin (Cho, K.W.Y. et al. 1991), was expressed at E6.5 in hgs -/-, indicating the presence of functional activin signaling. The co-precipitation of Smads 1, 2 and 4 with Hgs was only observed in the presence of cross linking agent (Miura, S. et al. 1999). This suggests that this association is transient or alternatively, these Smads may be in close proximity to Hgs but not associate with it. Furthermore, my observation that Hgs interacts with specific type I receptors, not including ALK4, does not support the model of Hgs acting to bring Smad2 to the activin receptor complex. The relevance of the interaction of Hgs with ALK1, ALK3, TßRI, and ALK6 is unclear. The loss of this association in 293T cells suggests that it may be mediated by COS-1 proteins that are not present in 293T cells. Perhaps, Hgs plays a role in trafficking of these receptors as it has been implicated in vesicular transport (Bean, A.J. et al. 1997; Komada, M. et al. 1997). The colocalization of
Hgs and SARA suggests the possibility that they may function together. However, overall, the results described in this study do not support the model of Hgs cooperating with SARA to recruit Smad2 to the activin receptor.
REFERENCES


