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REPRESSION OF TRANSFORMING GROWTH FACTOR BETA SIGNALING BY EPIDERMAL GROWTH FACTOR, RAS AND INTERFERON GAMMA

by

JACQUELINE DOODY

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

REPRESSION OF TRANSFORMING GROWTH FACTOR BETA SIGNALING BY EPIDERMAL GROWTH FACTOR, RAS AND INTERFERON GAMMA

by

Jacqueline Doody

Advisor: Professor David Foster

The transforming growth factor β (TGFβ) family of ligands is comprised of numerous members involved in a wide range of cellular processes including cell proliferation, growth inhibition, differentiation, wound healing and apoptosis. These processes are mediated by the action of ligand binding to serine/threonine kinase receptors that phosphorylate a signaling molecule, Smad, necessary for nuclear transmission of TGFβ and the related bone morphogenetic protein (BMP) signaling factor. Smad proteins bind to DNA directly or in conjunction with partner transcription factors to activate TGFβ and BMP-inducible genes. This thesis makes the determination that phosphorylation of Smads at their carboxy-terminal serines is responsible for Smad nuclear translocation and transcription activation since mutation of these serines abolishes these Smad activation events. Furthermore, phosphorylation of Smads by the serine/threonine kinase receptor is direct since the purified receptor can phosphorylate Smad *in vitro*. Smads therefore provide a link between receptor kinases and the nucleus.

This thesis additionally focuses on TGFβ and BMP-antagonistic signaling pathways and their repression of TGFβ signaling by targeting Smad proteins.

Epidermal growth factor (EGF) and other growth factors necessary for proliferation activate the signaling molecule, Erk, responsible for phosphorylating Smad at several serines in the middle of the Smad molecule. Phosphorylation of Smad by Erk results in a decrease of nuclear accumulation of Smad and thus inhibits acitvation of TGFβ– and BMP-responsive genes. . Moreover, overexpression of Ras, another signaling molecule for EGF, inhibits TGFβ signaling. Therefore one mechanism which may contribute to transformation of cells by oncogenic Ras is to prevent TGFβ growth inhibition by limiting Smad nuclear accumulation. Crosstalk between interferon γ (IFNγ) and TGFβ also involves inhibitory phosphorylation of Smad although this is not the principal mechanism of repression. IFNγ induces the expression of Smad7, an antagonist of the TGFβ-inducible signaling molecule, Smad3. Smad7 inhibits binding of Smad3 to the TGFβ receptors preventing activation of Smad3 in response to TGFβ. These results suggest that IFNγ and EGF use different mechanisms to reach the same goal: inhibition of TGFβ signaling.

Preface

I began working on the characterization of Smad1 in collaboration with Fang Liu and Marcus Kretzschmar, former postdoctoral fellows of Joan Massague's laboratory. During the investigation of crosstalk between TGFβ and the molecules EGF and IFNγ, I worked with Marcus Kretzschmar and Luis Ulloa, respectively. To distinguish the work that I was involved in from those of others on the same project, I cite the specific paper when discussing their results or showing their data. In this way both my contributions and theirs can be acknowledged without affecting the flow of the thesis.

Acknowledgments

I cannot begin to acknowledge the guidance and support I have received from professors, colleagues and friends throughout my years at Hunter College and Memorial Sloan Kettering Cancer Center. At Hunter College my thanks go to Hari and Shoba Bodduluri, Michael Hadjiargyrou, Peter Lipke, Kathi McDowell, Benjamin Ortiz, Geraldine Reichel and Rifka Rudner. At Memorial Sloan Kettering Cancer Center I have worked with and been encouraged by many friends which include Alejandro Zentella, Fernando Lopez-Casillas, Jeffrey Wrana, Rotraud Weiser, Francesc Ventura, Ermelinda Montalvo, Fang Liu, Inga Reynisdottir, Yan Luo, Akiko Hata, Marcus Kretzschmar, Luis Ulloa, YeGuang Chen, Susan Lee and David Leiden.

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Abbreviations

BG.....Betaglycan
BAMBI.....BMP and activin membrane-bound inhibitor
BMP.....Bone morphogenetic protein
CBP.....cAMP response element-binding protein
Co-Smad.....Common Smad
DAD.....Daughters against dpp
DPP.....Decapentaplegic
EGF.....Epidermal growth factor
EGFR.....Epidermal growth factor receptor
FGF.....Fibroblast growth factor
GS domain.....Glycine-serine domain
GFP.....Green fluorescence protein
HGF.....Hepatocyte growth factor
HAT.....Histone acetylase
HDAC.....Histone deacetylase
I-Smad.....Inhibitory Smad
IFNγ.....Interferon γ
IFNR1.....Interferon γ receptor 1
IFNR2.....Interferon γ receptor 2
LAP.....Latency-associated peptide
MH1.....MAD-homology domain 1
MH2.....MAD-homology domain 2
MAP.....Mitogen-activated protein
MAPK.....Mitogen-activated protein kinase
MAD.....Mothers against dpp
PTB domain. .....Phosphotyrosine binding domain
R-Smad.....Receptor-mediated Smad
RSK.....Receptor serine/threonine kinase
RTK.....Receptor tyrosine kinase
SARA.....Smad anchor for receptor activation
SBE.....Smad-binding element
SOS.....Son of sevenless
SH2.....Src homology 2
SH3.....Src homology 3
TGFβ.....Transforming growth factor β
TNFα.....Tumor necrosis factor α

Introduction

The transforming growth factor β (TGFβ) superfamily controls an array of functions including cell cycle progression, differentiation, adhesion, migration and extracellular matrix production (for review see Massague, 1990; Roberts and Sporn, 1993). TGFβ and related factors are critical components in determining cell fates during development and maintaining homeostasis and repair in adult tissues. These polypeptide growth factors have been found in virtually all tissues and encompass a large class of signaling molecules.

Remarkable progress has been made over the last several years elucidating the mechanism by which cells transmit the extracellular TGFβ signal to activate gene responses. A large body of work resulted in the understanding of how TGFβ binds to two related serine threonine kinase receptors, which in turn activate a downstream Smad signal transduction pathway. This thesis focuses on the characterization of Smad proteins: how they are activated by the receptor serine/threonine kinases (RSKs) leading to nuclear translocation and transcriptional activation of TGFβ family-responsive genes. Smad is not only shown to be a target for TGFβ signals it is also shown to be modulated in response to epidermal growth factor (EGF) and Interferon γ (IFNγ) which are antagonists of TGFβ and that these effects are inhibitory in nature. EGF inhibits Smad nuclear accumulation by phosphorylation while IFNγ upregulates the expression of an anti-Smad, Smad7. This particular inhibition by EGF may be one mechanism by which tumors escape growth inhibitory effects of TGFβ. Thus,

Smad receives opposing regulatory inputs through receptor tyrosine kinases (RTKs) and RSKs and this balance determines the level of Smad activity in normal and cancerous cells.

Ligand-receptor interactions in the TGFβ superfamily.

Production of activated ligand.

TGFβ was the first of a large family of growth factors discovered and is considered the prototype because of intense study and characterization (Table 1). The degree of identity between family members range from a high of 95% to a low of 20%. Some general characteristics define the family. TGFβ and related factors have 7-9 cysteines with conserved spacing between them that forms a characteristic cysteine knot when observed as a crystal structure (Daopin et al., 1992; Schlunegger et al., 1992; Griffith et al., 1996). TGFβ is synthesized by cells as a prohormone which is cleaved into a latency-associated peptide (LAP) that remains non-covalently associated with the bioactive TGFβ (Laurence et al., 1985; Pircher et al., 1986). Because of LAP association with TGFβ, TGFβ receptors are unable to bind to the growth factor. The physiological mechanism regulating latent TGFβ activation is not well understood though possibly the cell adhesion receptor $\alpha_v\beta_6$ integrin or thrombospondin-1 may be involved (Munger et al., 1999; Murphy-Ullrich and Poczatek, 2000). Bioactive forms of TGFβ and its family members form dimers, the pairing of which may be homo- or heterodimeric

Ligand	Type II Receptor	Type I Receptor	R-Smad	Co-Smad
TGFβ	TβRII	TβRI	Smad2 Smad3	Smad4 Smad4β
Activin	ActRII ActRIIB	ActRIB	Smad2 Smad3	Smad4 Smad4β
BMP2/4 BMP7	BMPRII ActRII ActRIIB	BMPRIA BMPRIB ALK2	Smad1 Smad5 Smad8	Smad4 Smad4β
Dpp[a]	*punt*	*thickveins*	*Mad*	*Medea*
60A[b]		*saxophone*		
daf7[b]	*daf4*	*daf1*	*daf8*	*daf14*
dbl1[b]	*daf4*	*sma6*	*sma2*	*sma4*

[a] *Drosophila* signaling pathway analogous to the vertebrate BMPs
[b] *C. elegans* pathways

Table 1. Molecular mediators of the TGFβ family signaling pathways. Known ligands and their signaling partners are listed for selected pathways. This is not a comprehensive list but a representative sample of the most well characterized ligands.

between subfamily members such as TGFβ1 and TGFβ2 (Cheifetz et al., 1987) or BMP4 and BMP7 (Aono et al., 1985).

Receptor complexes

TGFβ family members initiate their cellular action by binding to transmembrane receptors with intrinsic serine/threonine kinase activity. This receptor family consists of two subfamilies, type I and type II receptors (Table 1) which are structurally similar. Both have a cysteine rich extracellular domain, a short single-spanning transmembrane domain and an intracellular region mainly composed of a serine/threonine kinase, yet only type I receptors have a region rich in glycine and serine residues (GS domain) in the juxtamembrane domain (for review see Massague, 1998). Since the receptors can bind most ligands and other receptors to varying degrees it has been difficult to assign physiological partners to the various receptors.

Type II receptors autophosphorylate and are active on the cell surface (Lodish and Luo,1997; unpublished data) as a homodimer. Ligand binds directly to a receptor type II which, in the case of TGFβ and activin, helps recruit receptor type I homodimers into the complex (Wrana et al., 1992; Attisano et al., 1993; Yamashita et al., 1994) (Figure 1). Type I and type II receptors have intrinsic affinity for each other but it is believed the ligand adds stability to the complex (Ventura et al., 1994). BMPs can bind BMP receptor type I and type II separately at low affinity but association of the two receptors generates a high affinity

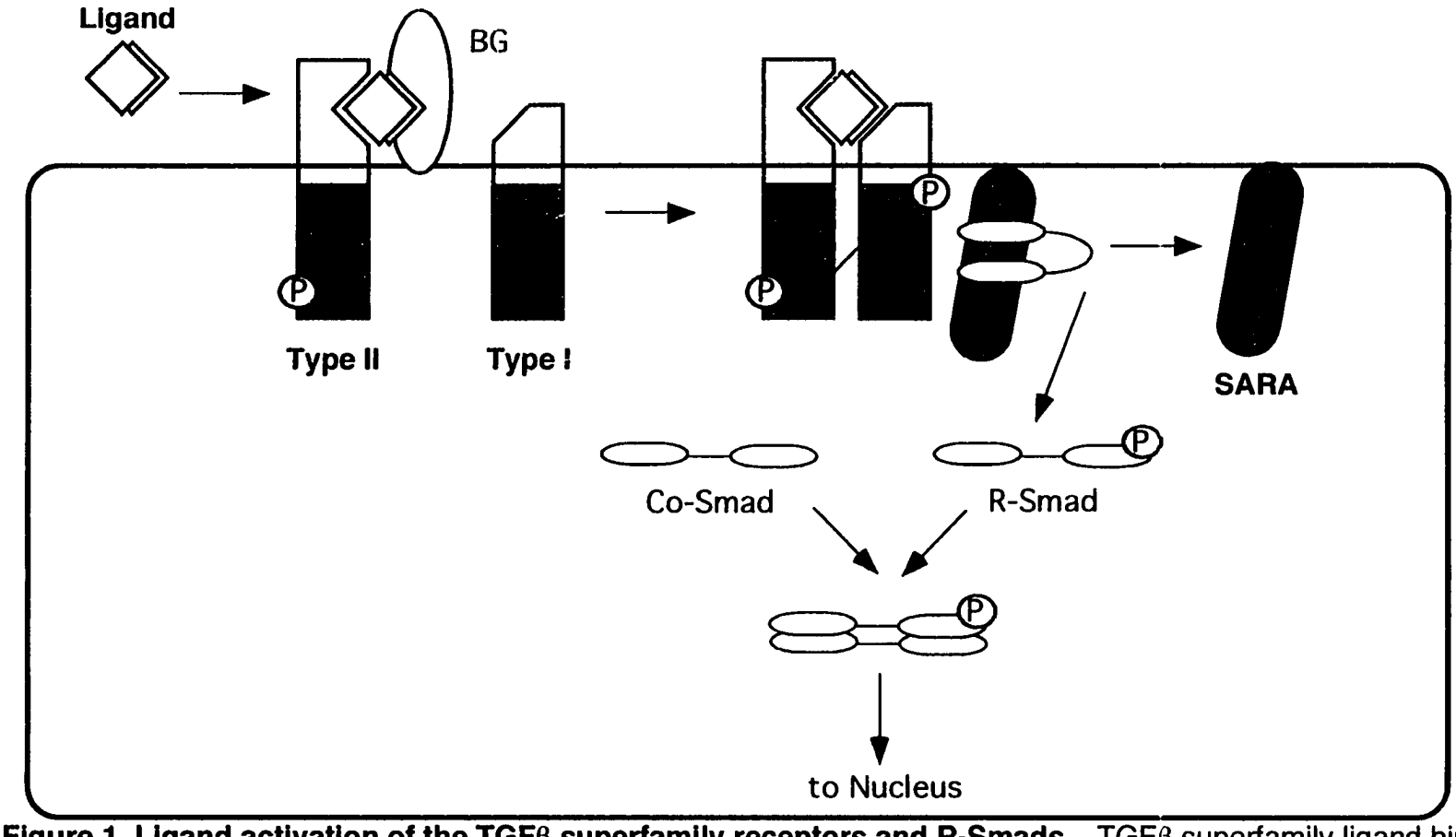

Figure 1. Ligand activation of the TGFβ superfamily receptors and R-Smads. TGFβ superfamily ligand binds to an autophosphorylated type II receptor with the assistance of betaglycan (BG). Upon ligand association, the type II receptor binds and phosphorylates the type I receptor in the GS domain (green shading). The activated type I receptor then phosphorylates the R-Smad which is sequestered to the membrane by the membrane-bound protein SARA. Phosphorylation of the R-Smad at a SSXS motif in the MH2 domain releases the autoinhibition of the two MH domains. The R-Smad is then free to heterdimerize with the Co-Smad and enter the nucleus.

complex (Liu et al., 1995). Once the ligand-type I-type II complex is formed, the activated type II receptor transphosphorylates the type I receptor in the GS domain to activate it (Wrana et al., 1994; Souchelnytskyi et al., 1996). The model predicts that type II and type I receptors act in sequence, which is supported by the finding that a constitutively active TGFβ type I is able to signal many TGFβ responses in the absence of TGFβ or TGFβ type II receptor (Wieser et al., 1995).

One of the TGFβ isoforms, TGFβ2, binds with low affinity to the TGFβ type II receptor and requires the cooperation of a third receptor, TGFβ receptor type III or betaglycan (Lopez-Casillas et al., 1991; Wang et al., 1991; Lopez-Casillas et al., 1993). Betaglycan (BG) is a membrane bound proteoglycan that stabilizes the TGFβ-receptor complex, but neither signals or contains a kinase region. BG also binds inhibin which antagonizes activin signaling (Lewis et al., 2000) therefore acting in different systems as either an agonist or antagonist to various signaling pathways. The other type III receptor, endoglin, may facilitate binding to the orphan receptor Alk1 (Johnson et al., 1996).

Smads

Genetic studies in *Drosophila* and *C.elegans* gave reveal the first insight into understanding how signals are transduced from the type I and type II receptors. In *Drosophila,* the gene *Mad* (*mothers against dpp*) was identified as a dominant enhancer of weak *decapentaplegic (dpp)* alleles, a *Drosophila* homologue of bone morphogenetic protein (BMP) (Raftery et al., 1995; Sekelsky

et al., 1995). In the same screen looking for maternal effect enhancers, a second mutation was located: *Medea,* (Raftery et al., 1995). Evidence that Mad is a downstream component of the dpp pathway came from the finding that Mad overexpression overcomes dpp-mutated eye phenotypes (Weirsdorff et al., 1996) and that Mad is required for cells to respond to dpp in visceral mesoderm and endoderm development (Newfeld et al., 1996). In *C. elegans*, *daf*1 and *daf*4 encode RSKs related to BMP receptors. By screening mutants which share the same phenotype with *daf4*, three genes were identified, *sma-2*, *sma-3* and *sma-4*, that showed homology to *Drosophila Mad* and *Medea* (Savage et al., 1996). Based on a shared mutant phenotype of small body size it was demonstrated that multiple *smas* are required in this signaling pathway. Nine gene homologues to *Mad* and *sma* have been identified in vertebrates (for review see Massague, 1998) and one additional in invertebrates (Brummel et al., 1999).

To simplify nomenclature, the designation Smad (for Sma/Mad related) is utilized to designate vertebrate homologues of *Sma* and *Mad.* These Smads fall into three categories: 1) receptor-mediated Smads (R-Smads) responsible for signaling from the RSKs to the nucleus, 2) common Smads (Co-Smads) that associate with the receptor-regulated Smads and 3) inhibitory Smads (I-Smads) that inhibit signaling by the other two groups of Smads (Figure 2a).

Structurally and functionally, Smads consist of three distinct domains. At the amino terminus is a highly conserved region, the MAD-homology domain 1 (MH1) present in all Smads except I-Smads. The carboxy terminal Mad-

A	Receptor Regulated		Common	Inhibitory
Vertebrates	Smad1	BMP		Smad6
	Smad5			
	Smad8		Smad4	
	Smad2	TGFβ/activin	Smad4β	Smad7
	Smad3			
Drosophila	MAD		MEDEA	DAD

B

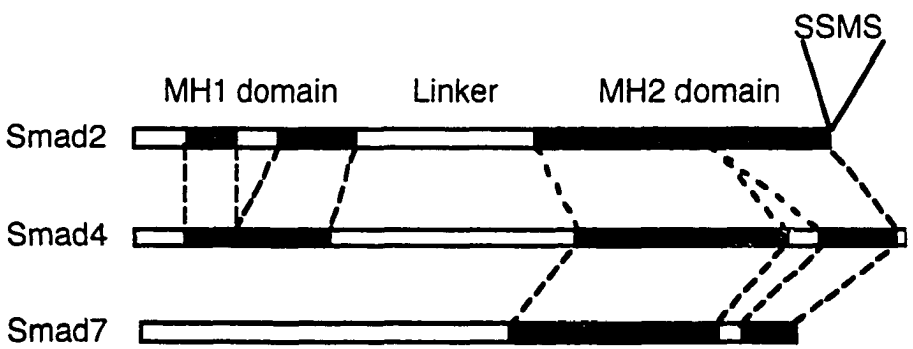

Figure 2. Summary of the Smad family. A. Smads are divided into three functional classes for vertebrates and *Drosophila*. Smad4 and Smad4β are common to all receptor-regulated Smads. **B.** The domain structure of representatives of pathway-regulated Smads (Smad2), common partner Smads (Smad4) and inhibitory Smads (Smad7). Areas of homology are indicated in black. The C-terminal SSXS phosphorylated by the receptor is illustrated in Smad2.

homology domain (MH2), present in all Smads, is connected to the MH1 domain by a proline-rich linker sequence Smad2 (Figure 2b). Since these proteins lack either a signal sequence or transmembrane domain and because Mad is found both in the nucleus and cytoplasm in *Drosophila* (Newfeld et al., 1996), they were proposed to function as intracellular mediators of RSK signaling.

Pathway-regulated Smads

Among the receptor-regulated Smads, the BMP subfamily is the largest. The BMP- or dpp-induced Smads consists of Smad1, Smad5 and Smad8 in vertebrates (Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Yingling et al., 1996; Chen et al., 1997c; Watanabe et al., 1997) and *Mad* in *Drosophila* (see above). Smad2 and Smad3 have been shown to be substrates for both TGFβ and activin receptors (Baker and Harland, 1996; Eppert et al., 1996; Graff et al., 1996; Marcias-Silva et al., 1996). In mammalian cells Smad2 and Smad3 are interchangeable in mediating growth inhibition and transcriptional activation of TGFβ and activin reporter genes (Lagna et al., 1996; Zhang et al., 1996) though Labbe et al. (1998) indicate Smad3 inhibits Smad 2 signaling. However, Smad3 knock-out mice were found to have an impaired TGFβ–induced growth inhibitory response (Datto et al., 1999) indicating Smad3 is necessary for some TGFβ signaling, Also, Smad3 functions as well as Smad2 in several transcriptional assays (unpublished data) demonstrating that in most cases Smad2 and Smad3 are indistinguishable.

The MH2 domain of the R-Smads appears to control most protein-protein interactions. The MH2 domain mediates association of the R-Smads into homodimers and consequently heterodimers with Co-Smads (Lagna et al., 1996; Zhang et al., 1996; Wu et al., 1997; Kawabata et al., 1998) (Figure 3). R-Smads exist as monomers which undergo homodimerization once activated (Kawabata et al., 1998). Activated homodimeric R-Smads then associate and form a heterodimeric complex with a Co-Smad which translocates to the nucleus and activates transcription (Lagna et al., 1996; Zhang et al., 1996) (Figure 4). Three residues within loop 3 of the MH2 domain crystal structure appear to mediate interactions with specific type I receptors (Chen et al., 1998). The L3 loop is invariant between the TGFβ- and activin-activated Smad2 and Smad3 while the BMP-activated Smad1, Smad5 and Smad8 differ at only 2 amino acids. For the RSKs, the L45 loop in the kinase is required for the correct identification of the corresponding R-Smad (Feng and Derynck, 1997; Chen et al., 1998). Association between the type I receptor and R-Smads is dependent on an active type I receptor kinase (Marcias-Silva et al., 1996; Nakao et al., 1997a). The association of a serine/threonine kinase receptor type I with an R-Smad leads to activation of the R-Smad via phosphorylation of the MH2 domain (Hoodless et al., 1996; Marcias-Silva et al., 1996; Kretzschmar et al,1997a; thesis). Phosphorylation is specific: Smads 1,5 and 8 are phosphorylated only by BMP type I receptors whereas Smad2 and 3 are phosphorylated specifically by TGFβ and activin type I receptors (Graff et al., 1996). The phosphorylation is direct

DNA Binding

Binding to Calmodulin

Inhibition of MH2

Nuclear localization signal

Smurf1 Binding

Association with Receptor

Binding to SARA

Inhibition of MH1

Smad Oligomerization

Association with transcription factors

Binding to HATs and HDACs

Transcriptional Activation

Figure 3. Functional characteristics of the Smad domains. A number of distinct functions can be attributed to each of the domains as indicated.

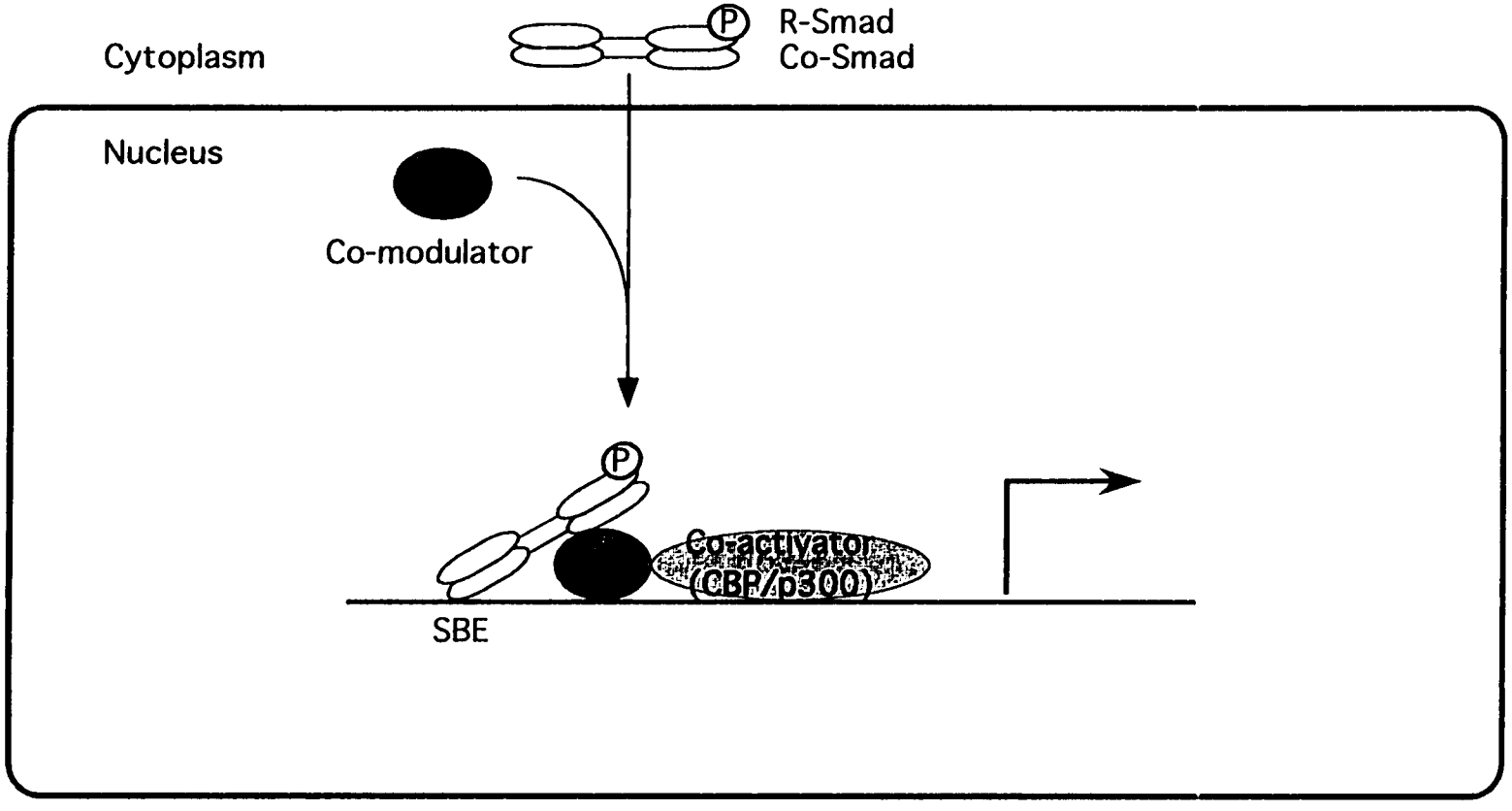

Figure 4. General model of transcriptional activation by R-Smads. The R-Smad-Co-Smad complex migrates to the nucleus, interacting with Co-modulators and Co-activators to regulate specific target genes. The Smads recognize a Smad binding element (SBE) which tethers either the R-Smad, Co-Smad or both to the DNA. Usually there is an adjacent element recognizable by the co-modulator which helps stabilize the complex on the DNA. A Co-activator is recruited which in turn recruits histone acetylates to facilitate transcription.

since purified BMP type I receptor can phosphorylate Smad1 *in vitro* (Kretzschmar et al., 1997a; thesis). Once phosphorylated, R-Smads translocate to the nucleus where they associate with DNA-binding factors through the MH2 domain (Chen et al., 1997b; Liu et al., 1997).

Recruitment of Smad2 and Smad3 to the TGFβ receptor is controlled by a membrane-bound protein termed Smad anchor for receptor activation (SARA) (Tsukazaki et al., 1998). This FYVE-containing protein is necessary for subcellular localization of inactivated R-Smads, bringing both the R-Smads and TGFβ type I together at the membrane for phosphorylation of the R-Smads. R-Smads associate with SARA in the MH2 domain. Subcellular localization is also controlled by the microtubules. Inactive Smads are sequestered to the microtubule network and are released upon TGFβ stimulation (Dong et al., 2000).

Once Smads are phosphorylated they dissociate from the receptors and SARA. Phosphorylation induces association with Smad4 and nuclear translocation of the complex. The role of phosphorylation in these events is not known, however it may relieve the inhibition of MH1 on the MH2 domain.

The MH1 domain in R-Smads and Co-Smads are homologous with Smad 2, containing a 30 amino acid insertion within the MH1 which differs from the other Smads. Initial work on Smads suggested that the MH1 domain functioned to inhibit activity of the MH2 domain through direct association (Hata et al., 1997). Indeed, overexpression of the isolated MH1 domain inhibited

TGFβ–induced association of Smad2 and Smad4. Phosphorylation of the C-terminal SSXS motif in R-Smads removes this inhibition.

In addition to repression of the MH2 domain, the MH1 domain is also responsible for the DNA-binding activity of the R- and Co-Smads. Binding of the MH1 domain to DNA was first described in the *Drosophila* Mad (Kim et al., 1997) and has been verified in vertebrate Smads as well. However, in Smad2 the insert within the MH1 domain interrupts the DNA-binding region and therefore Smad2 relies on Smad4 for DNA-binding ability when they are associated (Yagi et al., 1999). Crystallization of the MH1 domain of Smad3 in the presence of a DNA fragment containing two Smad binding elements (SBEs) reveals that DNA binding is accomplished by a β hairpin contacting DNA in the major groove (Shi et al., 1998). Smad2, which has an insert within its DNA-binding region, would appear to displace its β hairpin, providing evidence for its lack of DNA-binding. The DNA-binding activity of the MAD MH1 domain is inhibited by the MH2 domain implicating that MH1 and MH2 domains repress each other's function until activated.

The MH1 domain is also capable of binding transcription factors comparable to the MH2 domain. Proteins that have been reported to bind the MH1 domain include TE3, ATF2, Jun and Vitamin D receptor (Zhang et al., 1998; Hua et al., 1998; Sano et al., 1999).

Co-Smads

Smad4 was first characterized as a tumor suppressor gene in human pancreatic carcinomas (Hahn et al., 1996). Co-Smads do not associate with the RSKs nor do they become phosphorylated in response to ligand addition as the R-Smads do. However, Smad4 is necessary for Smad2 and Smad3 growth inhibitory effects (Lagna et al., 1996; Zhang et al., 1996). Smad4 resides in the cytoplasm where it associates with the activated R-Smads and the complex proceeds to the nucleus. Nuclear translocation of the R-Smads does not require Smad4 since Smad1 and Smad2 become nuclear in Smad4-deficient cells when activated (Liu et al., 1997). Support for these results is shown by the lack of a nuclear localization signal in the Smad4 MH1 domain present in the R-Smads (Xiao et al., 2000). Moreover, Smad4 is not required for Smad2-Smad4 complex binding to co-modulators in the nucleus. However, Smad4 is necessary for the complex to bind DNA and for transcriptional activation (Liu et al., 1997). Apparently, activated R-Smads carry Smad4 into the nucleus where Smad4 can bind DNA and activate transcription. Recently a second Smad4, XSmad4β, has been described in *Xenopus* (Howell et al., 1999; LeSueur and Graff, 1999; Masuyama et al., 1999). XSmad4β is interchangeable with XSmad4α (the homologue to human Smad4) in most functional assays except that XSmad4β can ventralize *Xenopus* embryos whereas XSmad4α has no effect. Interestingly, expression of Xsmad4α and Xsmad4β in *Xenopus* embryos is distinctly temporal since they appear at different stages of development.

Transcriptional activation

As described, R-Smads associate with Co-Smads after activation by the RSKs. The R-Smads-Co-Smads complex then proceeds to translocate to the nucleus by an unknown mechanism. Once nuclear, the complex binds to DNA, using the MH1 domain as a docking region (see above). Smads are also responsible for transcriptional activation, as evidenced by the use of an artificial GAL4-Smad1 fusion construct to activate a GAL4 reporter gene (Liu et al., 1996). GAL4-Smad1 activates transcription in response to BMP while GAL4-Smad2 activates transcription in a TGFβ-dependent manner. Both require Smad4 to do so (Liu et al., 1997).

The function of Smads may not be to target specific genes but to function as co-modulators. Smads appear to bind DNA with low affinity and specificity (Shi et al., 1998). As indicated, Smad2 has a disrupted DNA-binding region that inhibits its DNA-binding ability. Moreover, the consensus binding sequence of Smad3 and Smad4 is CAGA (Dennler et al., 1998; Zawel et al., 1998; Johnson et al., 1999) and it was shown that only one CAGA is necessary for each monomer of Smad3 to bind in a crystal structure analysis (Shi et al., 1998). A four base binding site is repeated too often in promoter regions to be of any value as a specific response element. Therefore, interactions with DNA-binding partners are probably necessary for Smads to function as specific transcriptional activators.

The first co-modulator described was Fast1, a forkhead/winged-helix DNA-binding protein found in *Xenopus* (Chen et al., 1996; Chen et al., 1997b).

Fast1 binds to an activin response element in the promoter region of the *Mix.2* gene. A ternary complex formed between Fast1, Smad2 and Smad4 (Chen et al., 1997b) sits on a short DNA motif encompassing both a required Fast1 DNA-binding site and a Smad binding element (SBE) (Zhou et al., 1998). Fast2, a mammalian homologue, is involved in *goosecoid* upregulation (Labbe et al., 1998) as well as regulation of the genes *lefty-2* and *nodal* (Saijoh et al., 2000). In all instances, either Smad2 (or Smad3) and Smad4 are required for transactivation though the Smad binding element which may differ somewhat from promoter to promoter. The Fast1 binding site remains invariant. Analysis of the complex shows Fast1 binds to the sequence-specific DNA element and along with Smad2, recruits Smad4, whose MH1 domain binds to an adjacent site which stabilizes the complex onto the DNA. SBE sites are often located near elements for other transcription factors such as AP-1, TFE3 and ATF2 in the TGFβ responsive PAI-1 promoter (Hua et al., 1998; Sano et al., 1999; Hua et al., 2000). Examples of physical interaction of Smads with other transcription factors include Smad with AML proteins, SIP1, OAZ, LEF1, Mixer and Milk (Verschueren et al., 1998; Germain et al., 2000; Hata et al., 2000; Labbe et al., 2000; Pardali et al., 2000). A recurrent theme is R-Smads and Smad4 cooperatively binding with a co-modulator to modulate gene expression. These co-modulators can be fork-head, homeobox or zinc finger transcription factors showing a diversity of cooperating co-modulators.

A consequence of Smad binding to DNA is the recruitment of transcriptional co-activators such as the cAMP response element-binding protein (CBP), p300 (Feng et al., 1998; Janknecht et al., 1998; Pouponnet et al., 1998; Shen et al., 1998; Topper et al., 1998). These co-activators enhance transcription by bringing SBEs in closer proximity to the basal transcriptional machinery and by acetylating histones, which result in decreasing chromosome condensation and facilitating transcription. Conversely, when Smad binds with a repressive transcription factor such as TGIF, histone deacetylases are recruited to condense chromosome and inhibit transcription (Wotten et al., 1999).

Alternatively, Smad and a co-modulator can bind DNA and act synergistically without interacting directly. STAT3 and Smad1 are brought together upon stimulation by BMP and leukemia inhibitory factor to associate with p300 and activate transcription (Nakashima et al., 1999). Both STAT3 and Smad1 bind to p300 without physically interacting with each other. Thus Smads binding to transcription factors insure 1) tighter binding to DNA; 2) increase specificity of the response; and 3) recruitment of co-activators. Since some of these co-modulating transcription factors are tissue-specific or induced by specific stimuli, Smads appear to have an important role in integrating signals from multiple origins and insuring a tight regulation of gene responses.

Other signaling molecules

In addition to Smads, other parallel pathways have been proposed to mediate TGFβ responses. Examples include TAK-1, a serine/threonine kinase of

the MAP (mitogen-activated protein) kinase kinase kinase family that is stimulated in response to TGFβ or BMP (Yamaguchi et al., 1995). Using a yeast two hybrid screen, it was found that XIAP, an inhibitor of apoptosis, binds BMP receptors and signals via TAB1 and TAK1 in a BMP-dependent manner (Yamaguchi et al., 1999; Shibuya et al., 1998). The Matsumoto laboratory has also shown that TAK1 is involved in activating the IL-1 signaling pathway, NIK-IκB, ceramide, Jnk and inhibiting β-catenin (Shirakabe et al., 1997; Ishitani et al., 1999; Ninomya-Tsuji et al., 1999: Takaesu et al., 2000), all of which are elements independent of TGFβ signaling except for possibly Jnk. ATF-2 is a nuclear target of Smad and the TAK1 pathway which may be how these two pathways converge, rather than at the receptor level (Sano et al., 1999).

Several WD domain proteins (STRAP, TRIP-1 and the Bα subunit of the phosphatase 2A) have been identified in two hybrid screens using TGFβ receptors (Chen et al., 1995; Choy and Derynck, 1998; Datta et al., 1998; Griswold-Prenner et al., 1998). Our laboratory has identified WD domain proteins during expression screenings using the TGFβ type I receptor, however aside from an affinity between the WD domain and the type I receptors, functional significance has not yet been observed. The role of these interactions in the mediation of TGFβ signaling remains unknown except for STRAP, which may have an inhibitory role (see below).

More compelling data exists providing evidence of interactions between TGFβ and BMP and various mitogen-activated protein kinase (MAPK) signaling

pathways. In developmental systems, BMP and RTK pathways have been shown to be antagonistic or synergistic depending on the system and cells observed. For example, in *Drosophila*, tracheal branching involves antagonistic interactions between EGF receptors and dpp (Wapper et al., 1997). However, EGF receptors and dpp synergize during *Drosophila* endodermal induction (Szuts et al., 1998). FGF antagonizes BMP in tooth and limb formation in mammals (Niswander and Martin, 1993; Ganan et al., 1996; Neubeser et al., 1997) while FGF and activin are both required for mesoderm induction in *Xenopus* (LaBonne and Whitman, 1994). These results indicate under different biological contexts RTK and RSK interactions can vary and that the interplay between these pathways is likely to be quite complex.

This interplay is borne out when evaluating the role of MAPKs in TGFβ signaling. Intensive study on the role of MAPK in TGFβ–mediated responses reveals conflicting reports. Erk1 is either activated (Hartsough and Mulder 1995; Mucsi et al., 1996; Axmann et al., 1998; deCaestecker et al., 1998; Lou et al., 2000) or not (Yan et al., 1994; Brown et al., 1999; Chatani et al., 1999; Engel et al., 1999; Hocevar et al., 1999; Hu et al., 1999; Iwasaki et al., 1999) depending on the cell type and length of response studied. Unfortunately, many labs utilize the 3TP-lux reporter construct as an indicator of TGFβ signaling which has several AP-1 sites included. EGF upregulates this construct in the absence of TGFβ (Carcamo et al., 1994). Now that other reporter constructs are available for measuring TGFβ-induced transcriptional activation these discrepancies can be

revaluated. p38 has also been implicated as a signaling molecule for TGFβ (Adachi-Yamada et al., 1999; Hanafusa et al., 1999; Iwasaki et al., 1999; Kimura et al., 2000;) although several laboratories see no activation of p38 by TGFβ (Mucsi et al., 1996; Atfi et al., 1997b; Hocevar et al., 1999; Hu et al., 1999). In some cases, when p38 activation by TGFβ is seen it is either Smad-independent (Hanafusa et al., 1999) or a late event (Adachi-Yamada et al., 1999). Further experimentation needs to be done to conclude if there is any role for p38 in TGFβ signaling.

The case for Jnk activation is stronger, with many reports showing elevated levels of Jnk 8-12 hours after TGFβ addition (Atfi et al., 1997a, 1997b; Engel et al., 1999; Hu et al., 1999; Wang et al., 1997; Zhou et al., 1999). Others observe a rapid activation of Jnk (Brown et al., 1999; Engel et al., 1999; Hocevar et al., 1999) while still others report no involvement of Jnk in TGFβ–induced gene expression (Mucsi et al., 1996; Hanafusa et al., 1999). These responses may be linked by TAK1 to TGFβ, which is known to activate Jnk (Zhou et al., 1999). Several reports have shown that rapid Jnk activation, if it exists, is independent of Smad (Engel et al., 1999; Hocevar et al., 1999).

Several TGFβ-responsive elements contain AP-1 sites. In artificial promoters AP-1 sites enhance TGFβ-dependent activation. It has been suggested that Smads might cooperate with AP-1 binding proteins to regulate activation of these AP-1 sites (Zhang et al., 1996). Jun and Fos transcription factors, which bind to AP-1 sites, may be important for this interaction since they

have been found to interact physically with Smads (Zang et al., 1998; Wong et al., 1999). Since Jun is a target for Jnk, convergence of TGFβ and Jnk signaling may be at the level of AP-1.

Inhibition of TGFβ signaling

The duration and intensity of TGFβ superfamily signaling are necessary for developmental systems and growth inhibition. BMPs and activin act as morphogens, inducing different sets of genes at different Smad levels where gradients are necessary for proper morphological developments. To achieve tight regulation, there needs to be both positive and negative mechanisms in place to allow fine-tuning of the signals. Moreover, negative regulation of TGFβ signaling provides crosstalk between other pathways and the TGFβ superfamily. Since there is a vast array of antagonistic stimuli to TGFβ, certain negative regulations are necessary to modify signaling in response to these stimuli. This may explain why there is such a vast array of negative regulation occuring at nearly every step in the TGFβ signal transduction pathway (Figure 5).

Inhibition of ligand binding to their receptors

As mentioned, TGFβ is synthesized as a prohormone requiring cleavage to obtain the mature activated ligand. However, the amino-terminal propeptide remains bound to TGFβ, inhibiting recognition by the receptor. Follistatin operates similarly to the propeptide, inhibiting recognition of activin by the activin

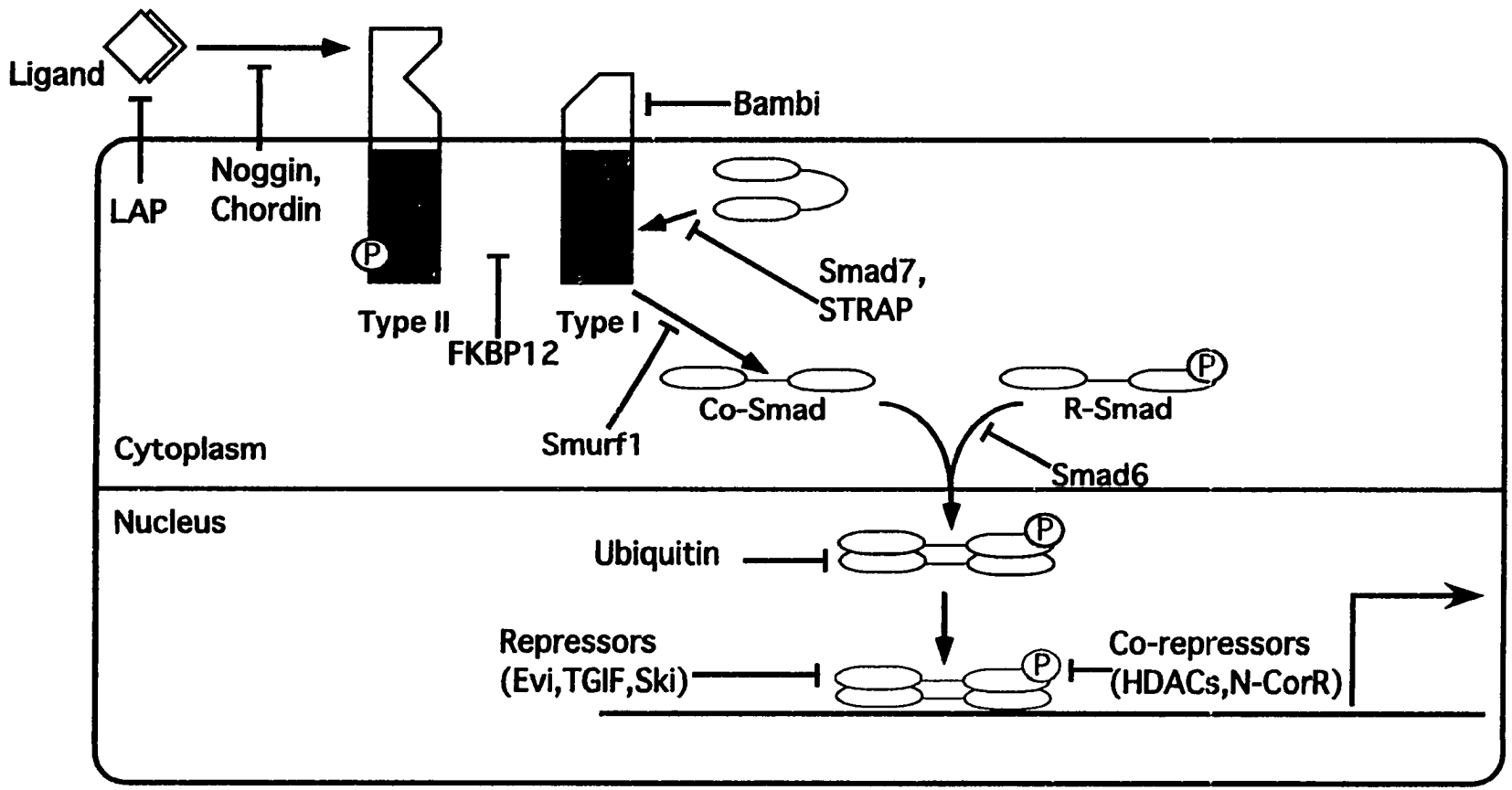

Figure 5. Negative regulators of the TGFβ superfamily pathway. There are negative regulators at nearly every level of the signaling pathway including negative feedback loops. The LAP fragment of the propeptide as well as ligand binding proteins such as noggin and chordin can prevent ligand binding to the receptors. Receptor-receptor and receptor-Smad interactions are inhibited by FKBP12,Bambi, Smad7 and STRAP while Smurf1 and Smad6 inhibit R-Smad-Co-Smad interactions. Within the nucleus, repressors such as Evi and TGIF recruit co-repressors to inhibit transcription.

receptors (deWinter et al., 1996) although it has also been observed to antagonize BMP (Sasai et al., 1995). That follistatin binds the heterodimer BMP4/7 is consistent with this observation (Yamashita et al., 1995). Four classes of proteins have been found to bind to BMP and prevent BMP binding to the BMP-type II receptor: follistatin, chordin, noggin and the DAN family of binding proteins (Sasai et al., 1994; Holley et al., 1996; Piccolo et al., 1996; Zimmerman et al., 1996; Hsu et al., 1998; Yokouchi et al., 1999; Piccolo et al., 1999). These proteins are expressed at varying times during development and are probably responsible for the tight temporal and spatial regulation of various processes. These four classes show no sequence homology and probably evolved independently.

Receptor regulation

BAMBI (BMP and activin membrane-bound inhibitor) is a transmembrane protein related to TGFβ type I receptors that lacks an intracellular domain (Onichtchouk et al., 1999). BAMBI inhibits BMP signaling during *Xenopus* development and has also been shown to inhibit TGFβ and activin signaling. Signaling is impaired by incorporation of BAMBI in type I heterodimers and interference with their activation.

Another regulator of type I receptors is FKBP12, a highly conserved intracellular protein involved in inhibiting the protein phosphatase calcineurin. FKBP12 binds to the GS domain of the type I receptor, blocking phosphorylation

of the type I receptor by the type II receptor (Wang et al., 1996; Chen et al., 1997a; Huse et al., 1999). Ligand-induced association of the two receptors releases FKBP12 which allows phosphorylation of the type I receptor. Association of FKBP12 to the type I receptor may be a mechanism which prevents spurious signaling in the absence of ligand.

Regulation of Smads

R-Smads and Co-Smads are responsible for growth factor-mediated signaling. A third Smad group, Smad6 and Smad7, also inhibits signaling (I-Smads) (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997b; Topper et al., 1997). Smad6 and Smad7 share sequence similarity in the carboxy-terminal MH2 domain with other Smads but their amino-terminal regions diverge significantly from the R- and Co-Smads as well as from each other. A *Drosophila* homologue, *Dad* (*daughter against dpp*), is induced by dpp although it blocks dpp activity (Tsuneizumi et al., 1997). The same holds true for Smad6 and Smad7. They are induced by the ligands which they will inhibit (Nakao et al., 1997b; Afrakhte et al., 1998; Nagarajan et al., 1999; von Gersdorff et al., 2000). Though both Smad6 and Smad7 have been shown to inhibit BMP and TGFβ signaling when overexpressed, Smad7 appears to target TGFβ and activin while Smad6 preferentially blocks BMP (Ishisaki et al., 1999). The mechanism by which they inhibit signaling is also slightly different. Smad7 associates with the TGFβ receptor type I, preventing association of Smad2 and Smad3 with the

receptor and thus blocking phosphorylation and activation of Smad2 and Smad3 (Hayashi et al., 1997; Nakao et al., 1997b). The WD protein, STRAP, stabilizes the association of Smad7 with the receptors, thereby assisting Smad7 in its inhibitory role of preventing R-Smads from docking (Datta and Moses, 2000). Smad7 exists normally in the nucleus but translocates to the cytoplasm upon TGFβ stimulation (Itoh et al., 1998).

Smad6 can also associate with the type I receptor but has only been in systems requiring Smad6 overexpression. At lower levels of Smad6, Smad6 forms BMP-dependent heterodimers with Smad1, preventing Smad1 from associating with Smad4 (Hata et al., 1998a). Without Smad4 as a partner, Smad1 is unable to signal a BMP-mediated response.

Smad6 and Smad7 expression levels increase in the presence of a ligand. Moreover, Smad3 and Smad4 binding sites are present in the promoter region of Smad7 (Nagarajan et al., 1999; vonGerdorff et al., 2000) while a Smad1, Smad5 and Smad4 binding motif is present in the promoter region of Smad6 (Ishida et al., 2000). In both cases, direct binding of the R-Smads and Co-Smads to the Smad binding element of the respective I-Smad is required for transcriptional activation. Therefore, inhibitory Smads may act as autoregulatory negative-feedback systems for TGFβ and related factors signaling pathways.

Targeting to the ubiquitin-proteasome pathway can regulate Smads. Smurf1, a member of the E3 ubiquitin ligases, preferentially induces Smad1 and Smad5 degradation with little effect on Smads 2,3 or 4, thereby targeting the

BMP pathway in *Xenopus* (Zhu et al., 1999). Smurf1 mediates the conjugation of ubiquitin to Smad1, insuring its degradation. By raising Smurf1 levels, BMP-dependent R-Smads protein levels decrease thereby lowering BMP-mediated responses and raising activin-mediated responses. E2 ubiquitin ligases have also been shown to target Smad2 mutants for degradation (Xu and Attisano, 2000), possibly a mechanism which increases tumorigenicity of cells with Smad defects. A third ubiquitin ligase has been shown to affect Smad2 degradation after TGFβ activation (Lo and Massague, 1999). Levels of phosphorylated Smad2 rapidly declined unless proteasome inhibitors were introduced. Ubiquinization of the R-Smads was not dependent on phosphorylation but instead was dependent on transport to the nucleus. This ubiquitin ligase is not Smurf1 since the required domain for ubiquinization is not the same as that needed for Smurf1 targeting of Smad1. Degradation of activated Smad in the nucleus may be a mechanism to rapily extinguish the TGFβ signal.

Transcriptional repression

Another mechanism by which Smad can be inhibited from activating TGFβ responsive genes is via direct binding to repressors which prevent Smad access to target promoters. Such is the case with the TGFβ signaling repressor Evi-1 (Kurokawa et al., 1998). Evi-1 interacts with Smad3 and prevents the formation of a Smad3-Smad4 complex on DNA. A similar scenario occurs with E1A where binding of E1A to Smad1, Smad2 and Smad3 prevents TGFβ–induced

transcription (Nishihara et al., 1999). However, in this instance, E1A interferes with TGFβ transcriptional responses by competing with Smad for binding to the histone acetylase (HAT) protein CBP/p300. The repressor need not bind to Smad to achieve inhibition of TGFβ –responses. In the case of the BF-1 transcription factor, it associates with Fast1, preventing the Fast1-Smad complex from occurring (Dou et al., 2000). Thus inhibition of TGFβ signaling can occur by either a repressor associating with Smad or to its co-modulator and preventing binding to DNA.

Besides acting as transcriptional activators, Smads can also function as repressors of transcription. The homeobox domain protein, TGIF, binds Smad2 and Smad3 in a TGFβ–dependent manner (Wotton et al., 1999a). This TGIF-Smad2 complex binds to TGFβ-responsive genes and repress their transcription. Repression by the TGIF-Smad2 complex is mediated by recruitment of histone deacetylases (HDACs) to the complex (Wotton et al., 1999a: Wotton et al., 1999b). The levels of TGIF in cells seem to determine how responsive the cell is to TGFβ signaling.

In a similar manner, several reports have shown an affinity between Smad2 and Smad3 with the repressors SnoN and Ski (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999; Sun et al., 1999a; Xu et al., 2000). Binding of Ski or SnoN to Smad2 or Smad3 represses transcription of several TGFβ target genes by recruitment of HDACs, notably the corepressor N-CoR (Akiyoshi et al., 1999; Luo et al., 1999; Stroschein et al., 1999). Upon TGFβ treatment, SnoN and

Ski levels decrease, becoming degraded by cellular proteasomes (Sun et al., 1999b). Apparently, Ski and SnoN repress TGFβ-responsive genes during the basal state to prevent spurious signaling but are rapidly degraded during TGFβ signaling. TGIF, Ski and SnoN all function by recruiting HDACs which deacetylate histones, thus inhibiting transcription. Transcriptional activators which associate with Smads recruit HATs, such as CBP/p300 to acetylate histones and enhance transcription. Apparently, TGFβ-induced responses are mediated by a balance between competing activators which recruit CBP/p300 and repressors which tether HDACs to Smad-responsive promoters. Thus, Smads interact with different DNA-binding factors which allow both positive and negative responses depending on the interacting partner.

Epidermal Growth Factor signaling

Growth inhibitory effects of TGFβ run counter to cellular proliferative effects seen in other growth factors such as EGF. EGF signals not only cellular proliferation but also cellular migration, cellular metabolism and cell survival. The ability of EGF to antagonize BMP signaling is seen in tracheal development in *Drosophila* while EGF inhibits BMP induction of osteogenesis in mammals (Bernier et al., 1992; Wappner et al., 1997). It would not be surprising to find instances of EGF inhibiting the TGFβ signaling pathway as a mechanism maximizing EGF responses.

EGF binds as a homodimer to the EGF receptor (EGFR). It is suggested that the EGFR occurs as a homodimer in unstimulated cells and that ligand binding stabilizes the complex, insuring the tyrosine kinase domains of the EGFR are in close proximity (Schlessinger, 2000). With the tyrosine kinases of EGFR homodimers adjacent to one another, their intrinsic tyrosine kinase domains are activated, leading to autophosphorylation as well as phosphorylation of specific substrates. Autophosphorylation sites on the EGFR serve as binding sites for SH2 (Src homology2) or PTB (phosphotyrosine binding) domains in numerous signaling proteins.

An example of a protein containing a PTB domain which promotes binding to the EGFR is Shc. Association of Shc with the EGFR permits tyrosine phosphorylation of Shc by EGFR or intracellular tyrosine kinases such as Src. This phosphorylation leads to binding of a second adapter protein, Grb2, via its SH2 domain to either Shc or EGFR itself. These adapter proteins utilize their SH2 and SH3 (Src homology 3) domains to mediate interactions between different proteins and facilitate phosphorylation of substrates. Grb2, for instance, binds the guanine nucleotide exchange factor Sos (son of sevenless) constitutively through its SH3 domain so that recruitment of Grb2 by the activated EGFR also recruits Sos. By Grb2 transporting Sos to the plasma membrane, Sos is able to stimulate the exchange of GTP for GDP on Ras leading to Ras activation (Bar-Sagi and Hall, 2000).

Once in the active GTP-bound state, Ras interacts with several downstream components such as Raf and PI-3 kinase. Raf is a MAPKKK at the head of a three kinase cascade (Davis, 1993). The minimal MAPK module is composed of three kinases that sequentially phosphorylate and activate the next component (Figure 6) (Garrington and Johnson, 1999). The first kinase in the module activated in response to EGF is the MAPKKK Raf. In turn Raf phosphorylates and activates the MAPKKs, Mek1 and Mek2, which are responsible for phosphorylating and activating the MAPKs, Erk1 and Erk2. Two other well defined MAPK kinase cascades utilize Jnk and p38 (Ip and Davis, 1998; Davis,2000). Though there is some crosstalk between modules it is believed that scaffolding proteins insure specificity (Garrington and Johnson, 1999). While MAPKKK and MAPK are serine/threonine kinases, MAPKKs are dual-specificity kinases able to phosphorylate their substrates both on tyrosine and serine/threonine residues (Widmann, 1999). Phosphorylation and activation of MAPK leads to phosphorylation of both cytoplasmic and nuclear proteins as MAPK translocates to the nucleus. Thus EGF signaling utilizes a myriad of components to transmit its signal to the nucleus.

Interferonγ signaling

Other antagoniss of TGFβ signaling are interferon γ (IFNγ) and cytokines, all of which are necessary for immune responses to external assaults. TGFβ is an immunosuppressor (for review see Letterio and Roberts, 1998) involved in

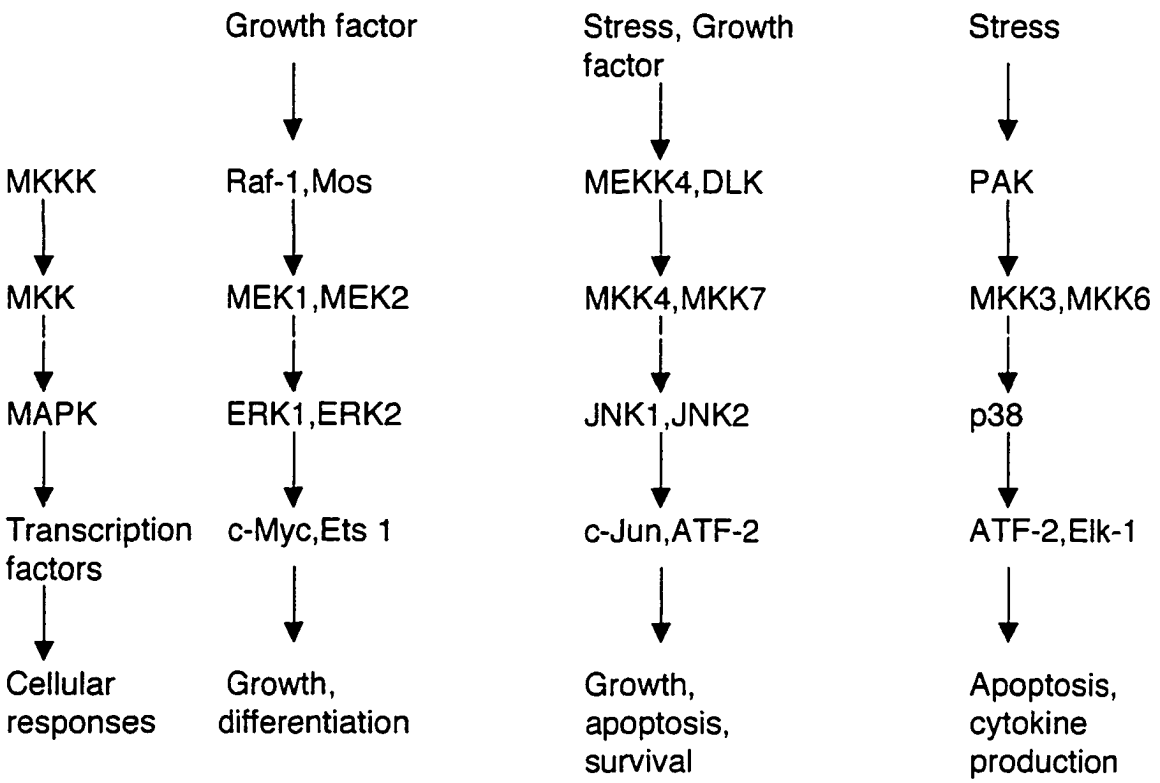

Figure 6. Structure of MAP kinase signal transduction pathways.

Extracellular signals such as growth factors or stress initiate ligand binding to their receptors. These receptors are responsible for activated MKKKs, which in turn activate one or several MKKs. The activated MKKs phosphorylate MAPK, which can then phosphorylate transcription factors necessary for the cellular responses triggered by the growth factor.

differentiation, proliferation and inactivation of many different immune cells (Bauvois et al., 1992; Schmitt et al., 1994; Xiao et al., 1996) while IFNγ mediates antiviral responses and induces upregulation of the immune system.Interestingly, TGFβ and IFNγ are both well known to inhibit cell growth though by differing mechanisms.

IFNγ is induced by immune and inflammatory stimuli and produced by T cells and natural killer cells (Bach et al., 1997). IFNγ binds as a homodimer to the IFNγ receptor (IFNR1) which, in turn, induces dimerization of the receptor (Figure 7). In unstimulated cells, IFNR1 associates with the tyrosine kinase molecule Jak1 in the intracellular domain of the receptor which remains after dimerization of the receptor. Once IFNR1 complexes with IFNγ, a second receptor, IFNR2, can bind and it is believed that IFNR2 is necessary for stabilization of the ligand-receptor complex. IFNR2, like IFNR1, has no intrinsic kinase activity but has an associated tyrosine kinase molecule, Jak, bound to the intracellular region. In the case of IFNR2, Jak2 binds to a four residue sequence in the carboxyl tail of the receptor.

Once a stabilized complex of ligand, receptors and Jak proteins has formed, Jak1 and Jak2 are sequentially activated by auto- and trans-phosphorylation. Jak2 transphosphorylates itself on tyrosines and is required for subsequent tyrosine phosphorylation and activation of Jak1. Stimulation of the IFNγ pathway requires activation of both Jaks, where the sole function of the

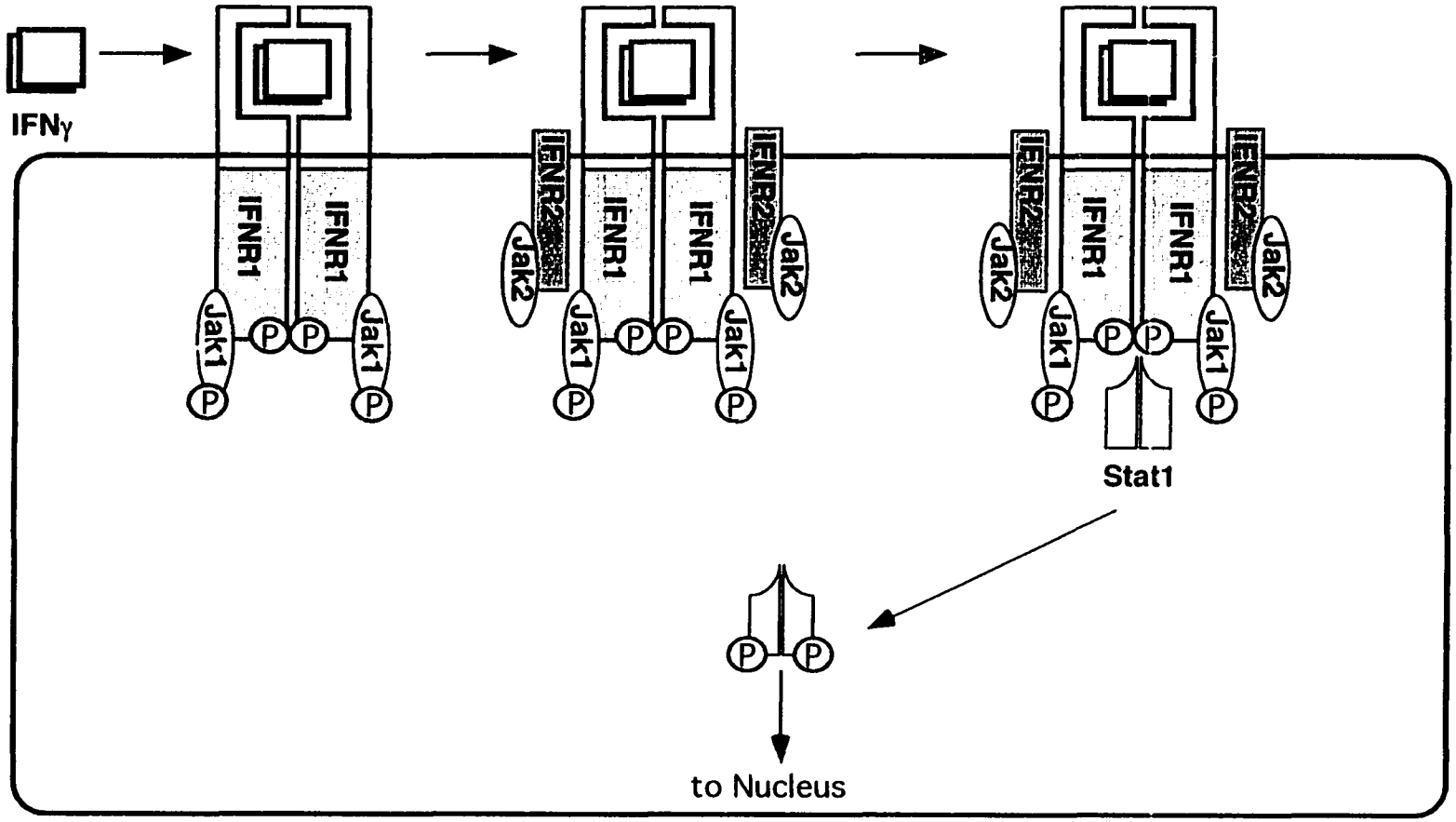

Figure 7. Signaling via the IFNγ pathway. IFNγ induces homodimerization of the IFNR1 leading to the association with IFNR2. The receptor complex allows transphosphorylation of the preassociated Jaks. Stat1 docks to the tyrosine phosphorylated IFNR1, which in turn is phosphorylated. Once dimerized, Stat1 migrates to the nucleus and transcriptionally activates IFNγ–inducible genes.

receptors is to bring the Jaks into close proximity to allow activation after ligand binding (Stark et al., 1998). Jaks are also responsible for phosphorylating a tyrosine residue near the carboxyl-terminus of IFNR1, forming a docking site for Stat1.

Two Stat1 proteins bind to the docking sites of the IFNR1 dimer via an SH2 domain located in the center of the Stat1 molecule. This brings Stat1 into close proximity with Jaks which phosphorylate Stat1. Tyrosine-phosphorylated Stat1 dissociates from the receptor and forms a homodimeric complex which translocates to the nucleus. At some point after tyrosine phosphorylation by Jak, Stat1 homodimers are phosphorylated at a serine residue by an as-yet-undefined MAP kinase-like enzyme which enhances its activity. Upon translocating to the nucleus, Stat1 binds to IFNγ-inducible genes to stimulate transcription either alone or in combination with other transcription factors (Chatterjee-Kisbore,2000).

Materials and Methods

Cell Lines and Transfections

COS-1 and Mv1Lu cells were obtained from American Type Culture Collection. Monkey COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfection of COS-1 cells was as previously described (Ausubel et al., 1987), adding the indicated plasmid to DMEM containing 10% NuSerum (Collaborative Research), 400μg/ml DEAE-dextran and 100μM chloroquine. Twenty-four hours later, cells were trypsinized and reseeded in 6 well dishes for the various assays or slides for immunoflourescence. The cell line R1B (subclone L17) is a derivative of the mink lung epithelial cell line Mv1Lu chemically mutagenized to render it TBRI defective (Boyd and Massague, 1989; Laiho et al., 1990). The Mv1Lu cell line is maintained in minimal essential medium (MEM) supplemented with 10% FBS and non-essential amino acids (NEAA). R1B (L17 subclone) cells are cultured in MEM-NEAA minus histidine medium and selected with 10% dialyzed FBS and 100μg/ml histidinol. Both the parental Mv1Lu and derived R1B cells are transfected using the DEAE-dextran method. Briefly, exponentially growing cells are incubated in MEM containing the various constructs as indicated; 100mM chloroquine and 125μg/ml DEAE-dextran for three hours. After shocking cells for two minutes with 10% dimethyl sulfoxide in phosphate-buffered saline (PBS) cells were washed and returned to MEM with 10% FBS.

U4A and U4A/Jak1 cells were a kind gift from G. Stark and I. Kerr. U4A is a human fibroblast cell line developed to analyze the IFNγ signaling pathway and is Jak1-deficient (Stark et al., 1998). A subclone was developed with a stably expressed Jak1, UYA/Jak1 (Muller et al., 1993) to dissect out the signaling molecules for IFNγ. Both cell lines are cultured in DME containing 10%FBS, with 50μg/ml hygromycin to maintain the Jak1 construct in the U4A/Jak1 subclone. U4A and U4A/Jak1 cell lines are transfected by the DEAE-dextran method as outlined above for Mv1Lu cells.

The mouse mammary epithelial cell line, EpH4, and its v-Ha-Ras transformed derivative, EpRas (Oft et al., 1996), are a generous gift of E. Reichmann. Both cell lines are maintained in DMEM containing 10mM Hepes buffer and 8% FBS with 500μg/ml G418 to select for the Ras construct in EpRas cells. Lipofectamine (Life Technologies) was utilized for transient transfections, overlaying cells for six hours before adding media and 20% FBS. Twenty-four hours later, the lipofectamine solution was replaced with fresh media and cells reseeded for the various assays.

Colon cancer cell lines were kindly provided by N. Rosen. HT-29, Colo 205, LoVo and DLD1 cells required RPMI 1640 media (Life Technologies) with 10% FBS and are transfected with lipofectamine as described above for EpH4 and EpRas cells using Opti-MEM1 (Life Technologies) to optimize transfection.

Reagents

Except where specified, the factors BMP-2 (5nM, Genetics Institute), TGFβ-1 (100pM, R&D Systems), EGF (18nM, R&D Systems), TNFα (20nM, R&D Systems) and IFNγ (500IU ml^{-1}, Boehringer Mannheim) were used. Where indicated, cells were treated with the MEK1 inhibitor PD98059 (100μM, New England Biolabs) or the PI(3) kinase inhibitor wortmannin (0.1μM, Calbiochem) for 1 hour before addition of growth factors. Recombinant, activated Erk2 (New England Biolabs) was utilized in the *in vitro* phosphorylation experiments.

Plasmids

Backbones for the various mutant constructs are the full length Smad1, Smad2 and Smad3 cDNA clones ligated into the pCMV5 vector (Andersson et al., 1989) with a Flag epitope encoded at the 5' end (DYKDDDK) for mammalian cell expression. The same mutant constructs were prepared for bacterial expression by subcloning into a pET11 expression vector (Novagen) encoding an N-terminal hexahistidine tag. Mutant plasmids were obtained by PCR using standard *in vitro* mutagenesis procedures (Ausubel et al., 1987).

Assessing transcriptional and functional activity of various Smad constructs, reporter plasmids were generated. GAL4-Smad was constructed by fusing Smad1 to the GAL4 DNA-binding domain (1-147) (Ptashne, 1988; Sadowski and Ptashne, 1989) and subcloned into the vector pCS2 (a gift of R. Harland). The GAL4-Smad1 plasmid is cotransfected with a GAL4-luciferase

reporter construct (Gu et al, 1997) to assess transcriptional activity. The luciferase reporter construct A3-Luc was subcloned from a core promoter with 3 AREs previously described as A3-CAT (Huang et al, 1995) into the pGL2-Basic luciferase vector (Promega) and 32F-lux (Lukas et al., 1997) kindly provided by A. Flattaey. Other plasmids employed include the constitutively activated BMPR-1B(Q203D), H-Ras ^{V12} in pCS2, FAST-2 (Liu et al., 1999), constitutively active MEK1 from S. Mansour and GFP (Clontech).

Antibodies

Anti-Smad2 and anti-Smad3 polyclonal antibodies were raised in rabbits by immunization with the recombinant linker region of human Smad2 (aa183-273) and human Smad3 (aa143-231) which, because they are highly homologous and crossreact, are referred to as anti Smad2/Smad3. These antibodies were affinity purified with immobilized Smad2 or Smad3. Rabbit polyclonal antibodies raised against a peptide corresponding to amino acids 457-467 of Smad2, including the phosphorylated serine residues at positions 457 and 467 (Upstate Biotechnology), were used in Western immunoblotting of carboxyl-terminus phosphorylated Smad2. Human Smad2-specific antibodies raised against a peptide encompassing residues 81-107 and Smad3-specific antibodies against residues 192-211 of human Smad3 (Zymed) were used in Western immunoblotting. Flag-tagged proteins were visualized with the monoclonal antibody M2 (Eastman Kodak).

Phosphorylation assays

In vivo phosphorylation was performed in cells labeled with 1mCi ml $^{-1}$ [^{32}P] orthophosphate for two hours in phosphate-free medium. After incubation with various factors, cells were lysed in 50mM Tris-HCl pH7.4, 150mM NaCl, 5mM EDTA, 25mM NaF, 1% Triton-X-100 in the presence of protease and phosphatase inhibitors. Immunoprecipititated protein was separated on SDS-PAGE and transferred to PVDF membrane. Immunoprecipitated Smads were either visualized by autoradiography or subjected to further digestion. Membranes pieces were incubated with trypsin (Promega) in 50mM ammonium bicarbonate and resolved on 16% Tris-glycine gels and subjected to autoradiography. For phosphoamino acid analysis, membrane pieces are partially hydrolyzed in 6M HCl and separated in two dimensions using the HTLE-7000 system (CBS Scientific).

Smad constructs in pET expression vectors were purified for *in vitro* kinase assays. Briefly, bacterially expressed Smads were purified on a Ni-NTA column (Qiagen) after lysing the cells in 20mM Tris-HCl pH7.5, 500mM NaCl, 10mM immidazole, 10% glycerol, 0.1% NP40 in the presence of protease inhibitors. Equivalent amounts of recombinant Smad protein, as quantified by Coomassie staining, were incubated with recombinant, activated BMPR-1B(Q203D) cytoplasmic domain or recombinant, activated Erk2 in 50mM Tris-HCl pH7.3, 100mM NaCl, 10mM $MnCl_2$, 10% glycerol, 5mM dithiotreitol and 0.05% Triton-X-100. [γ^{32}P] ATP was added in the presence of 0.3mM ATP for 20

min at 28°C. Reactions were stopped by addition of buffer containing 6M guanidinium-HCl and Smad protein recovered with Ni-NTA agarose.

[^{35}S] Metabolic labeling, Immunoprecititation and Western blot analysis.

Cells were washed in methionine-free media and incubated for two hours in methionine-free media supplemented with 100μCi/ml[^{35}S]-Translabel (Amersham). Cells were lysed in 50mM Tris-HCl pH7.4, 150mM NaCl, 1% Triton-X-100 in the presence of protease inhibitors. M2 antibodies were used for immunoprecititations of Flag-tagged Smads or polyclonal antiserum for the endogenous Smads as indicated above. Purification of the desired Smad was realized by passing the lysed material over a 1:1 ProteinA:ProteinG Sepharose column, resolved by SDS-PAGE and visualized by autoradiography. Western immunoblotting consisted of running the lysed proteins on SDS-PAGE and probing with the indicated antibody before subjecting to chemiluminescence (ECL,Amersham).

Immunofluorescence assays.

24 hours after transient transfection, cells were plated on slides and allowed to adhere for an additional 24 hours. Fixation of the proteins was accomplished with a 1:1 solution of methanol and acetone and cells were subsequently permeabilized with 0.2% Triton-X-100 in PBS. Transfected Smads were visualized with M2 anti-flag antibodies followed by an FITC-conjugated anti-

mouse immunoglobulin antibody (Jackson Immunoresearch). Endogenous Smads were prepared by a triple sandwich method (Harlow and Lane, 1988) with affinity-purified anti-Smad antibodies, followed by a biotin-conjugated anti-rabbit immunoglobulin antibody and finally visualized using streptavidin conjugated to FITC. All slides were counterstained with DAPI (Sigma) to visualize cell nuclei (data not shown). In all cases, at least 100 stained cells were scored.

Reporter assays

Cells were transiently transfected with the A3-Luc reporter construct and treated with growth factors 18 hours before assaying for luciferase activity (Promega). Cells were washed with PBS and lysed in 120μl of lysis buffer (Luciferase assay system, Promega). After 10 min cells were scraped and centrifuged. Luciferase activity was measured in the first 20 sec after substrate addition using a luminometer (Berthold Lumat LB9501).

Reporter affinity labeling assays.

TGFβ1 was labeled with ^{125}I and cross-linked to TGFβ receptors *in vivo* by incubation with disuccinimidyl suberate (Pierce) as previously described (Cheifetz et al., 1990). Briefly, cells were incubated with iodinated TGFβ for two hours in binding buffer containing media and 25mM Hepes pH7.5 followed by a 15min incubation with 0.3mM DSS at room temperature. Cells were solubilized and the resulting cell extracts were subjected to immunoprecipitation with anti-

Flag antibodies. Labeled receptors in the immunoprecititates and total cell lysates were visualized by SDS-PAGE and autoradiography.

Results

Characterization of Smad1

In addition to searching for possible candidates as TGFβ receptor type I substrates, Dr. Joan Massague asked me to help Dr. Fang Liu characterize Smad1, a homolgue of *Drosophila* Mad, recognized as a BMP signaling molecule. Expertise in phosphorylation assays enabled me to help pinpoint the location of Smad phosphorylation by BMP receptor I, along with Dr. Marcus Kretzschmar. Additionally, I developed an immunofluorescence assay to monitor Smad nuclear translocation. These experiments are described below.

All experiments referred to in these figures were performed by me except for those referred to in Figs. 12, 16, 17 and 20, where I was responsible for constructing the mutants used to produce the data. Data presented were included in the following two papers:

Liu, F., Hata,A., Baker,J.C., Doody,J., Carcamo,J., Harland,R.M. and J.Massague (1996). A human Mad protein acting as a BMP-regulated transcriptional activator. Nature, 381: 620-623.

Kretzschmar, M., Liu,F., Hata,A., Doody,J. and J.Massague (1997). The TGF-β family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes Dev., 11: 984-995.

Smad1 is a signaling molecule for BMP

Genetic studies in *Drosophila* and *C.elegans* gave us the first clues to understanding how signals are transduced from the serine/threonine kinase receptors of the TGFβ superfamily to the nucleus. The product of the *Drosophila* gene *Mad* (*mothers against dpp*) and *Medea* were identified in a genetic screen for dominant enhancers of weak *dpp* alleles (Raftery et al.,1995, Sekelsky et al.,1995). Mad is a downstream component of the dpp pathway as is evidenced by rescue of *dpp* mutants (Wersdorff et al., 1996). Furthermore, Mad is required for dpp response (Newfeld et al., 1996). In *C. elegans*, screening for mutants which mimic serine/threonine kinase receptor mutants revealed three genes, *sma2, sma3* and *sma4*, which are components of the TGFβ superfamily signaling pathway (Savage et al., 1996). Mad and sma were the first molecules cloned which are downstream elements of the TGFβ superfamily pathway. To study the involvement of these Mad family members in vertebrate BMP signaling, we cloned a human homologue of Mad which shows 91% identity with the *Drosophila* Mad over the N- and C-terminal domains (Liu et al., 1996). We designated this protein Smad1 (for *Sma* and *Mad* homologue). Utilizing *Xenopus* embryo animal cap assays which ventralizes upon addition of BMP, we were able to show that Smad1 mimics BMP signaling by also enhancing ventralization of these embryo animal caps. Therefore, Smad1 probably participates in BMP signaling.

To further investigate the role of Smad1 in BMP signaling, a Flag epitope was first introduced on the N-terminus of Smad1 (Fig.8). This construct was transfected into R-1B/L17 mink lung epithelial cells which can be made responsive to TGFβ, activin or BMP by transfection of the appropriate receptors (Carcamo et al.,1994; Liu et al.,1995). Flag-Smad1 transfected alone into these cells contained basal phosphorylation, as determined by precipitation from [^{32}P] phosphate-labeled cells through the Flag epitope tag (Fig.9,lane1). The phosphorylation level of the transfected Smad1 was increased when co-transfected with the BMP receptors BMPR-IA and BMPR-II and cells were stimulated with BMP2 (Fig.9). Phosphorylation of Smad1 occurs rapidly, within 10 min after BMP2 addition and is sustained for at least 60 min indicative of a substrate for a signaling pathway. This phosphorylation by BMP2 is not mimicked by either TGF-β or activin (Kretzschmar et al., 1997a) implying that Smad1 is specifically activated by BMP.

Since Smad1 lacks a signal sequence or transmembrane domain and Mad was found in both the nucleus and cytoplasm in *Drosophila* (Newfeld et al., 1996) it was decided to investigate Smad1 cellular localization. Using COS-1 cells, which respond to BMP without transfecting in the BMP receptors, Flag-Smad1 was transfected and visualized by immunofluorescence using anti-Flag antibodies. Smad1 was present predominantly in the cytoplasm but translocated to the nucleus upon addition of BMP2 (Fig.10). Nuclear localization of Smad1 was maximal 30-60 min after BMP2 stimulation indicating that Smad1 may

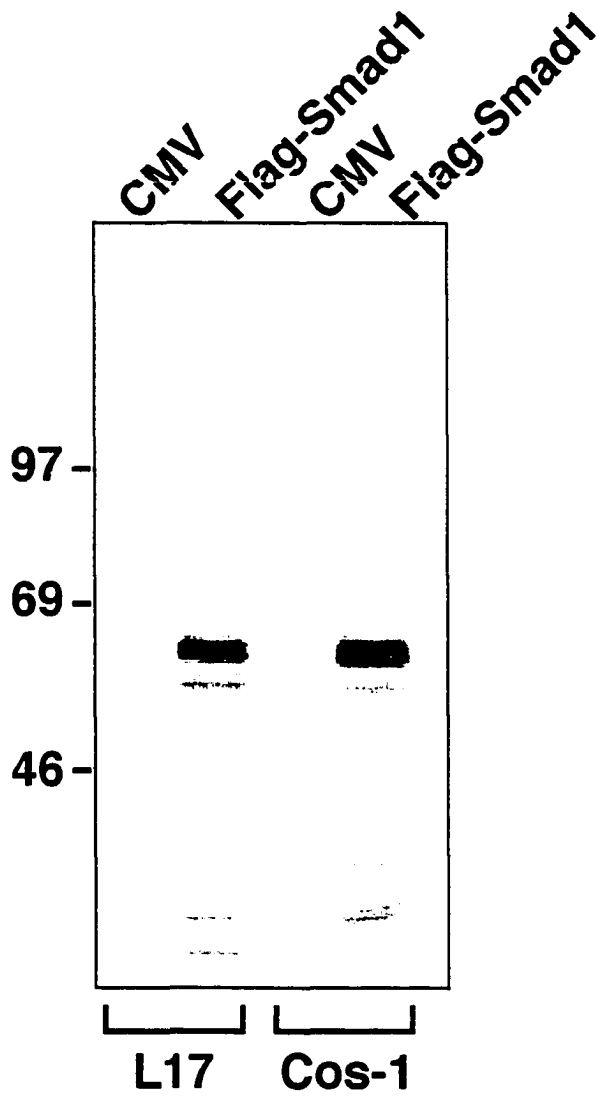

Figure 8. Expression of Flag-Smad1 in COS-1 and R1B/L17 cells. COS-1 and R1B/L17 cells were transfected with a Flag-tagged Smad1 construct and subjected to Western immunoblotting with anti-Flag antibodies.

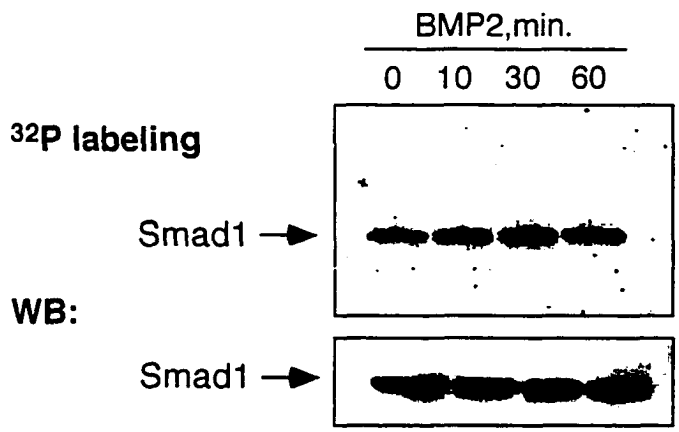

Figure 9. BMP-induced phosphorylation of Smad1. R1B/L17 cells were cotransfected with Flag-tagged Smad1 and BMP receptors and labeled with ^{32}P. Cells were incubated with BMP2 for the times indicated before lysis. Flag-Smad1 was immunoprecipitated with anti-Flag antibodies. Smad1 expression was controlled by anti-Flag immunoblotting.

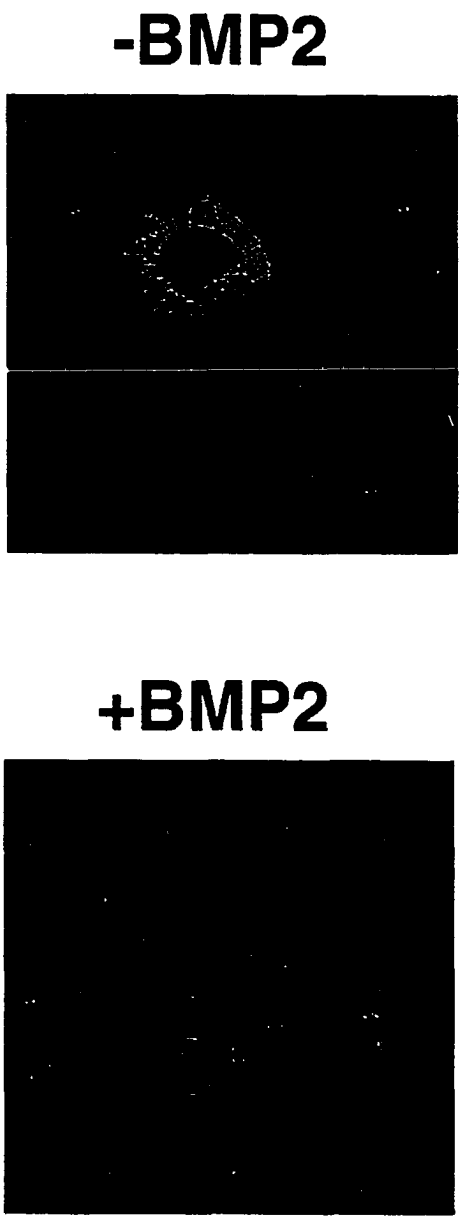

Figure 10. Nuclear localization of Flag-Smad1 in COS-1 cells. COS-1 cells were transfected with Flag-Smad1. 5nM BMP2 was added where indicated 30 min before immunostaining with M2 anti-Flag monoclonal antibodies and FITC-conjugated secondary antibodies. The same slides were counterstained with DAPI to visualize nuclei.

function downstream of the BMP receptors and convey BMP2 signals to the nucleus. To show that activation of Smad1 leads to potential transcriptional activation by BMP, a GAL4-Smad1 construct was generated by fusing the GAL4 DNA-binding domain to Smad1 (Liu et al., 1996) and transfected into R1B/L17 cells together with a CAT construct containing a GAL4-binding site. If Smad1 has any transcriptional activity CAT levels would increase. CAT activity increased significantly only when the BMP receptors were co-transfected along with stimulation by BMP but not under any other conditions, showing that Smad1 is a transcriptional activator and that this activation occurs only after stimulation by BMP. Therefore, BMP leads to phosphorylation and subsequent translocation of Smad1 to the nucleus where it acts as a transcriptional activator of presumably BMP-regulated genes.

Direct phosphorylation of Smad1 by BMP receptor type I

To characterize further the phosphorylation of Smad1 in response to BMP, a phosphoamino acid analysis is used to determine whether the phosphorylation occurs on serine, threonine or tyrosine. *In vivo* phosphorylation of Smad1 occurred overwhelmingly on serine with some phosphothreonine when cells were activated by BMP2 (Fig.11), ruling out any direct phosphorylation by a tyrosine kinase. Since the receptors for BMP are both serine/threonine kinase receptors, they may be responsible for the direct phosphorylation of Smad1. Previously it was shown that the type II receptor for TGFβ directly phosphorylates and

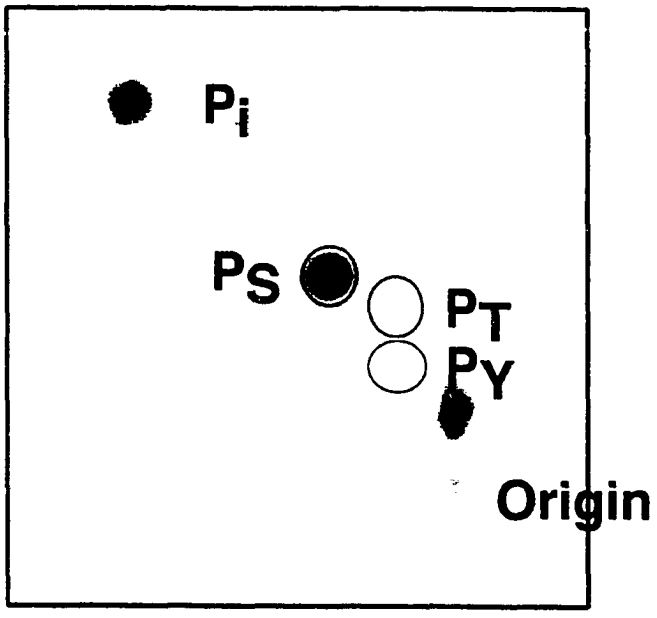

Figure 11. Phosphoamino acid analysis of *in vivo* Smad1 phosphorylation. ^{32}P-labeled Flag-Smad1 from cells treated with BMP2 was subjected to HCl digestion and two-dimensional phosphoamino acid analysis. The areas where the various phosphoamino acids migrate are indicated with circles: Phosphoserine (P_S), phosphothreonine (P_T) and phosphotyrosine (P_Y). P_i = inorganic phosphate.

therefore activates the type I receptor (Wrana et al.,1992). The TGFβ receptor type I is then responsible for activation of the various downstream targets of TGFβ (Wrana et al.,1992). The same holds true for the BMP receptors where BMPRII phosphorylated BMPRI, which then activates the various BMP responses (Liu et al.,1995). Therefore, BMPRI would probably be the receptor to phosphorylate Smad1 if a direct phosphorylation occurs. Recombinant BMPRIB(Q203D), which carries an activating mutation (Weiser et al., 1995), was purified and assayed *in vitro* with a purified bacterially expressed Smad1 to determine whether Smad1 is phosphorylated by the BMP receptor-associated kinase. With increasing amounts of BMPRIB(Q203D) increased amounts of Smad1 phosphorylation is observed (Fig.12). In contrast, an equivalent amount of activin receptor type I did not phosphorylate Smad1 over background (Kretzschmar et al., 1997a) indicating that BMPRI phosphorylation of Smad1 is specific and direct. Therefore, BMPRI-1B has intrinsic Smad1 kinase activity that is proportional to the level of *in vivo* signaling activity of this receptor.

BMP-dependent phosphorylation of Smad1 is on the C-terminus

To determine the site at which the BMP receptor type I phosphorylates Smad1, the region was mapped by analysis of tryptic fragments. Tryptic digestion of Smad1 results in numerous small fragments and a large polypeptide that encompasses most of the linker region (Fig.13). When [^{32}P] labeled Flag-Smad1 from BMP2 treated cells was trypsinized there were two

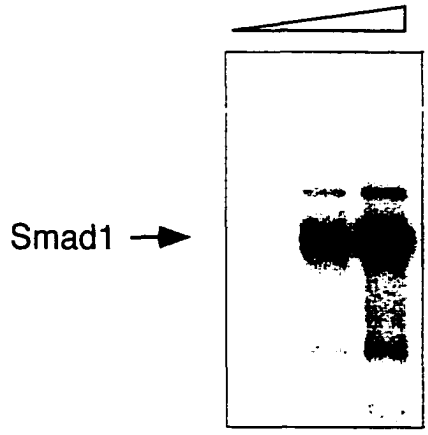

Figure 12. Smad1 is phosphorylated by BMP receptor type 1. Purified, recombinant Smad1 protein was tested at concentrations in the nanomolar range as a substrate for recombinant activated BMP type I receptor kinase (BMPR-IB (Q203D)).

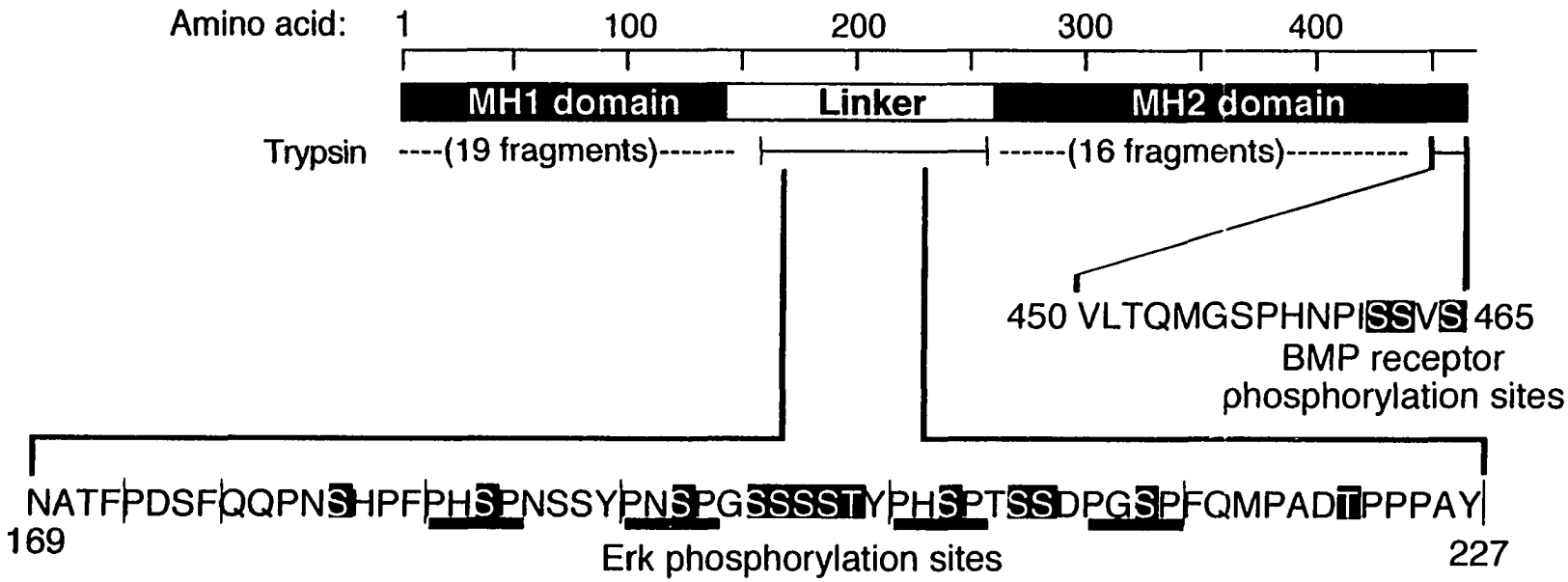

Figure 13. Predicted proteolytic digestion patterns of Smad1. The partial sequence of the linker region containing potential phosphorylation sites is shown. Residues mutated singly or in groups are highlighted, and Erk consensus sites are underlined. The sequence of the C-terminal tryptic peptide is also listed with the BMP receptor phosphorylation sites highlighted.

phosphorylated bands corresponding to the linker and a small fragment which could originate either in the MH1 or MH2 domain of Smad1 (Fig.14). However, non-treated cells showed only linker domain phosphorylation (data not shown). These data would indicate that the BMP receptor type I phosphorylates Smad1 in the MH1 or MH2 domains. Since certain mutations in the carboxy-terminal domains of Smad1 and Smad2 can inhibit agonist-induced phosphorylation (Eppert et al., 1996, Hoodless et al., 1996), specifically the GWG sequence (aa419-421), attention was focused on the MH2 domain. Smad1 is directly phosphorylated by BMP receptor I, Smad2 and Smad3 associate with and are phosphorylated by TGFβ (Marcias-Silva et al., 1996; Zhang et al., 1996) but Smad4 does not get phosphorylated by the various growth factors (Zhang et al., 1996). Initial attempts to identify the sites of Smad1 phosphorylation located serines associated with Smad1, Smad2 and Smad3 but lacking in Smad4 (Fig.15). The serine closest to the GWG of the carboxyl-terminus of Smad1 is located at residue 431. An alanine mutation of serine 431 had little effect on BMP-induced Smad1 phosphorylation (data not shown). Another area having serines specific to signaling Smads, but absent in Smad4, is at the C-terminal tail (SSXS). A Flag-Smad1 mutant construct substituting alanine for serine at amino acids 462-465 (AAVA) was transfected with and without BMP receptors into R1B/L17 cells. There was no increase in phosphorylation of Flag-Smad1 by BMP in the mutant protein compared to wild type (Fig.16; compare lane 3 to lane5) suggesting that BMP receptor I predominantly phosphorylates Smad1 at the C-

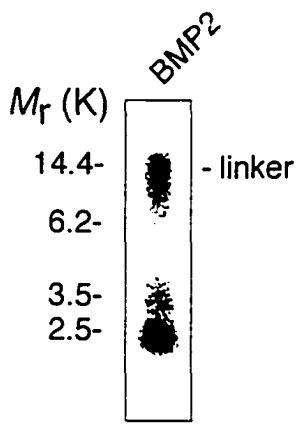

Figure 14. Tryptic digestion of Flag-Smad1 shows phosphorylation of two regions when stimulated with BMP. Trypsin digests of [^{32}P]-labeled Flag-Smad1 from BMP2-treated cells .Phosphorylation of the linker is attributed to the effect of serum factors in the medium.

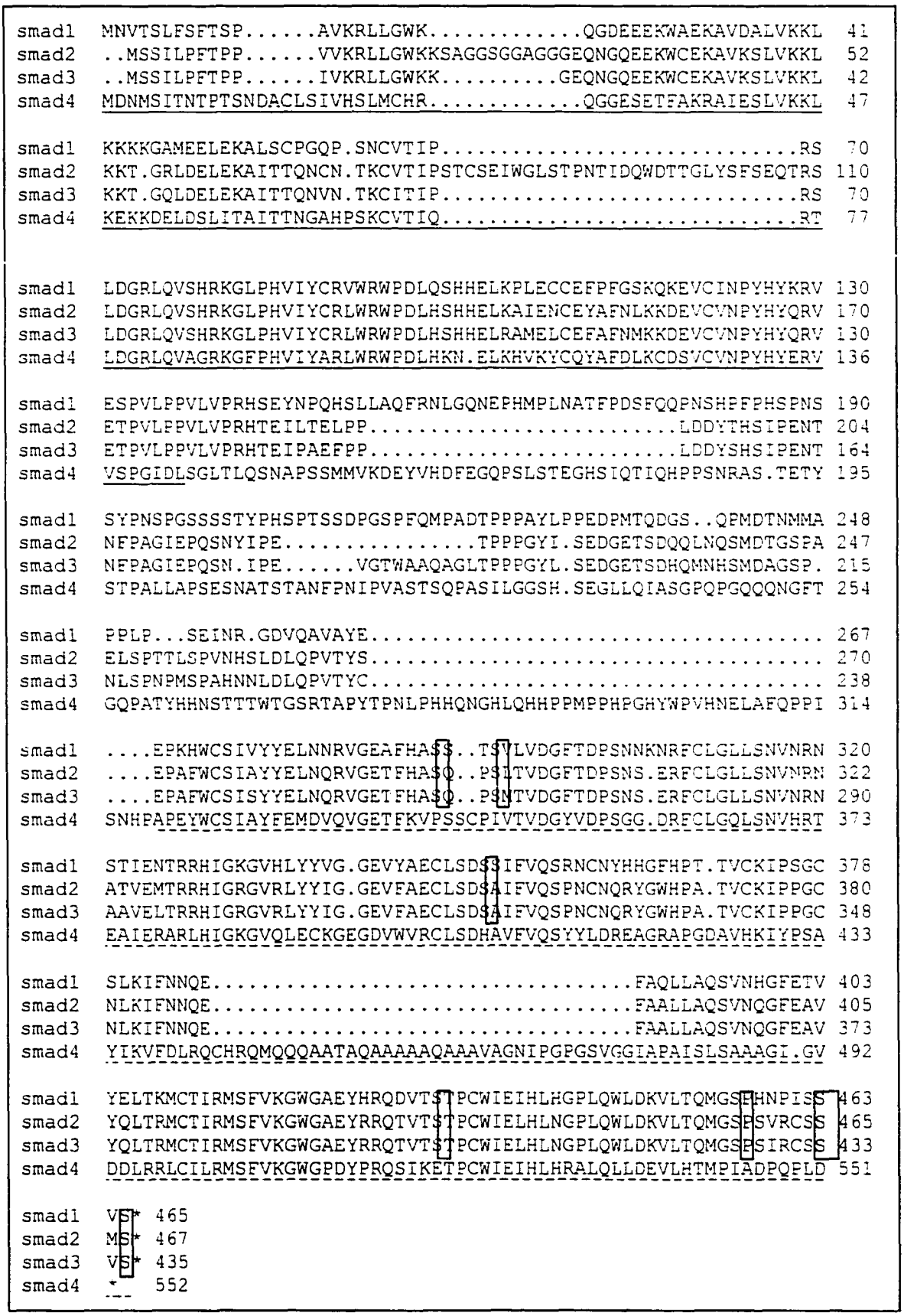

Figure 15. Sequence comparison of mammalian Smads. Alignment of the amino acid sequence of human Smad1, Smad2, Smad3 and Smad4. The MH1 domain (solid underline) at the amino-terminus and the MH2 domain (dotted underline) at the carboxyl-terminus are linked by a proline-rich region. Conserved serines from Smad1, Smad2 and Smad3 but lacking form Smad4 are boxed in the MH2 domain. Gaps introduced to maximize alignment are shown as dots and the amino acid residues are numbered on the right.

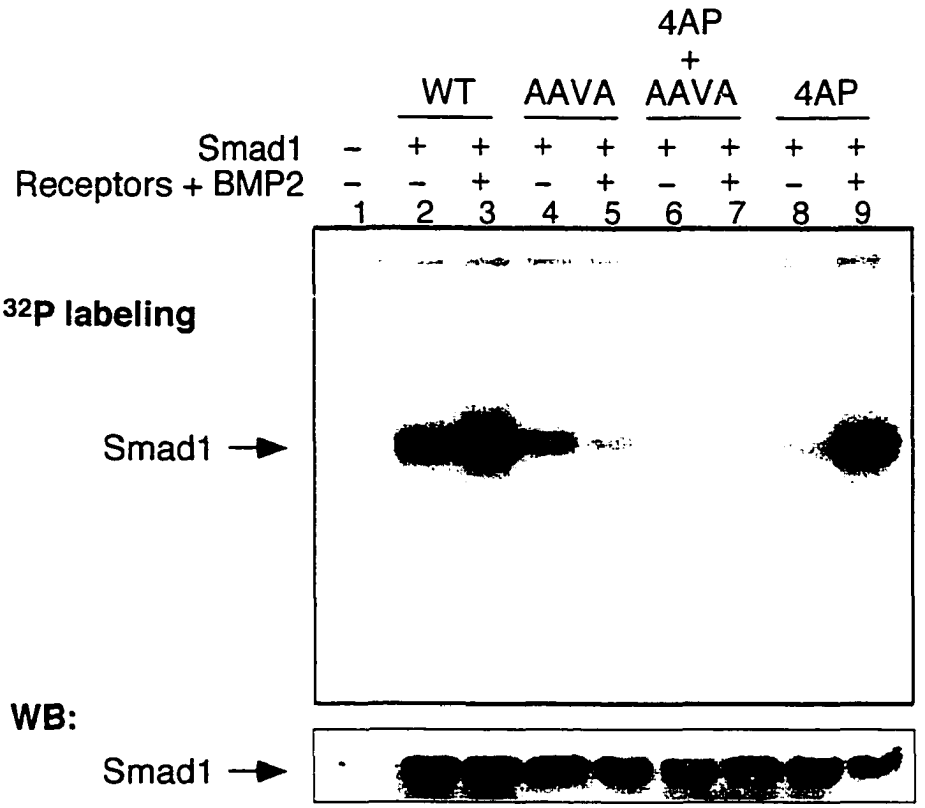

Figure 16. BMP-dependent and -independent phosphorylation of the wild-type (WT) Smad1 and selected mutants in transfected R-1B/L17 cells. BMP stimulation was provided by co-transfection of BMP receptors with Flag-Smad1 followed by BMP2 addition 20 min before cell lysis. Smad1 expression was controlled by anti-Flag immunoblotting. Mutants shown contain mutations of SSVS to AAVA in carboxyl-terminus, and PXSP to PXAP (4AP) in the linker region.

terminal serines. Attempts to determine which and how many of the three carboxy-terminal serines are phosphorylated in response to BMP have failed to yield conclusive results. Phosphoamino acid analysis of single serine to threonine mutations of the SSVS motif has revealed only little or no increase in the amount of phosphothreonine in response to BMP. While replacing serine with threonine may not result in effective threonine phosphorylation, it does not interfere with phosphorylation of the remaining two serine residues. In contrast, alanine substitution for any of the serines at the C-teminal tail abolishes all BMP-induced phosphorylation (data not shown). Conceivablly, the alteration of a single residue deforms the delicate three-dimensional structure of Smad1 thus preventing phosphorylation of the remaining serines.

BMP-independent phosphorylation is localized to the linker region.

As mentioned previously, there are two regions where Smad1 is phosphorylated: the linker region and the very C-terminal SSVS. Even thought the carboxy-terminal tail SSVS motif is phosphorylated in response to BMP, the linker region is as yet uncharacterized. As shown, most of the phosphorylation of Smad1 is on serines with some threonines and no tyrosines (Fig.11) therefore mutation of serine and threonine residues to alanine and valine, respectively, were constructed to determine the sites of phosphorylation in these mutants. Analysis of the mutants in transfected cells demonstrated that none of the mutants had a strong effect on BMP-induced phosphorylation (Fig.17, data not

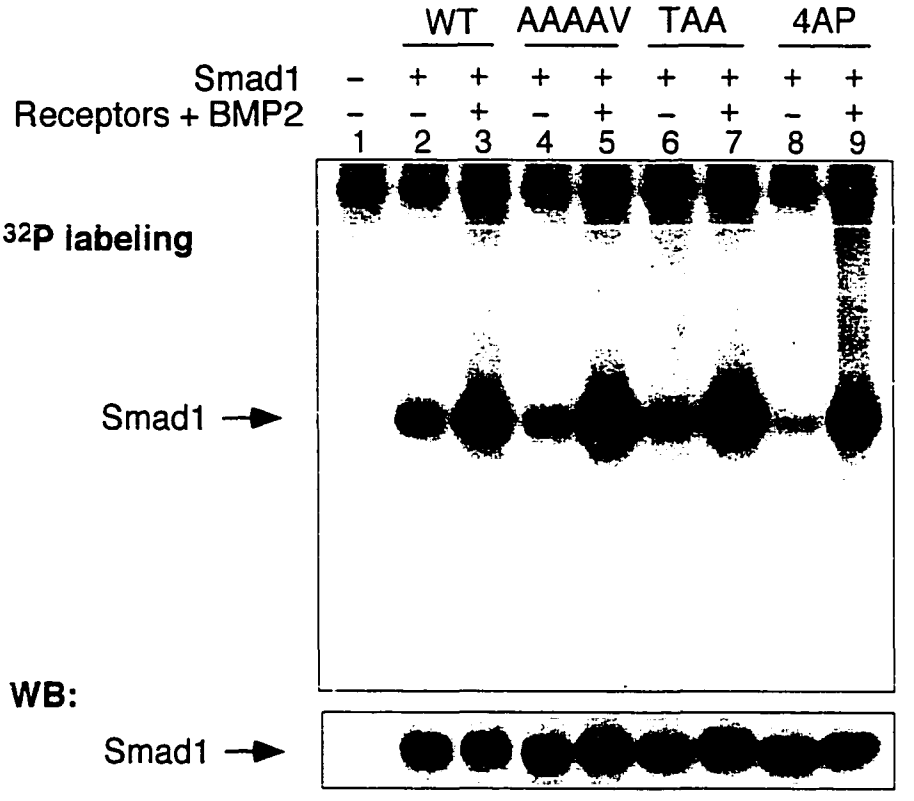

Figure 17. Basal and BMP-induced phosphorylation of the wild-type (WT) Smad1 and selected mutants in transfected R-1B/L17 cells. BMP stimulation was provided by co-transfection of BMP receptors followed by BMP2 addition 20 min before cell lysis. Smad1 expression was controlled by anti-Flag immunoblotting. The Smad1 mutants shown contain mutations of S to A or T to V in amino acids 198-202 (AAAV), 209-210 (TAA) or all four PXSP motifs (serines 187,195,206 and 214) (4AP).

shown). However, the mutant Smad1(4AP), which has mutations in four repeated PXSP motifs, shows a strong reduction in basal phosphorylation (Fig.16, lanes 8 and 9). Mutation of fewer PXSP motifs gave a proportional reduction in phosphorylation (data not shown) suggesting that all four sites are phosphorylated in the wild-type Smad1. When the PXSP motifs of the linker region and the C-terminal SSVS were mutated simultaneously, phosphorylation of Smad1 was completely lost, regardless of the presence or absence of BMP stimulation (Fig.16, lanes 6 and7). This indicates that the PXSP motifs are phosphorylated in the linker region and that they are phosphorylated independently of the C-terminal SSVS motif phosphorylation. Furthermore, the phosphorylation of the linker region is performed by a kinase other than the BMP receptor.

Smad1 is activated by phosphorylation of the C-terminal SSVS motif but not the linker region

To determine the functional significance of the various phosphorylation sites of Smad1, it was necessary to ascertain whether the mutants would hinder nuclear translocation. As previously shown, Smad1 is distributed mainly in the cytoplasm in the absence of BMP and becomes mostly nuclear when cells are stimulated with BMP (Fig.18,19). In contrast, BMP does not induce nuclear accumulation of Smad1 containing the SSVS to AAVA mutation indicating that the loss of phosphorylation at the C-terminus prevents BMP-induced nuclear

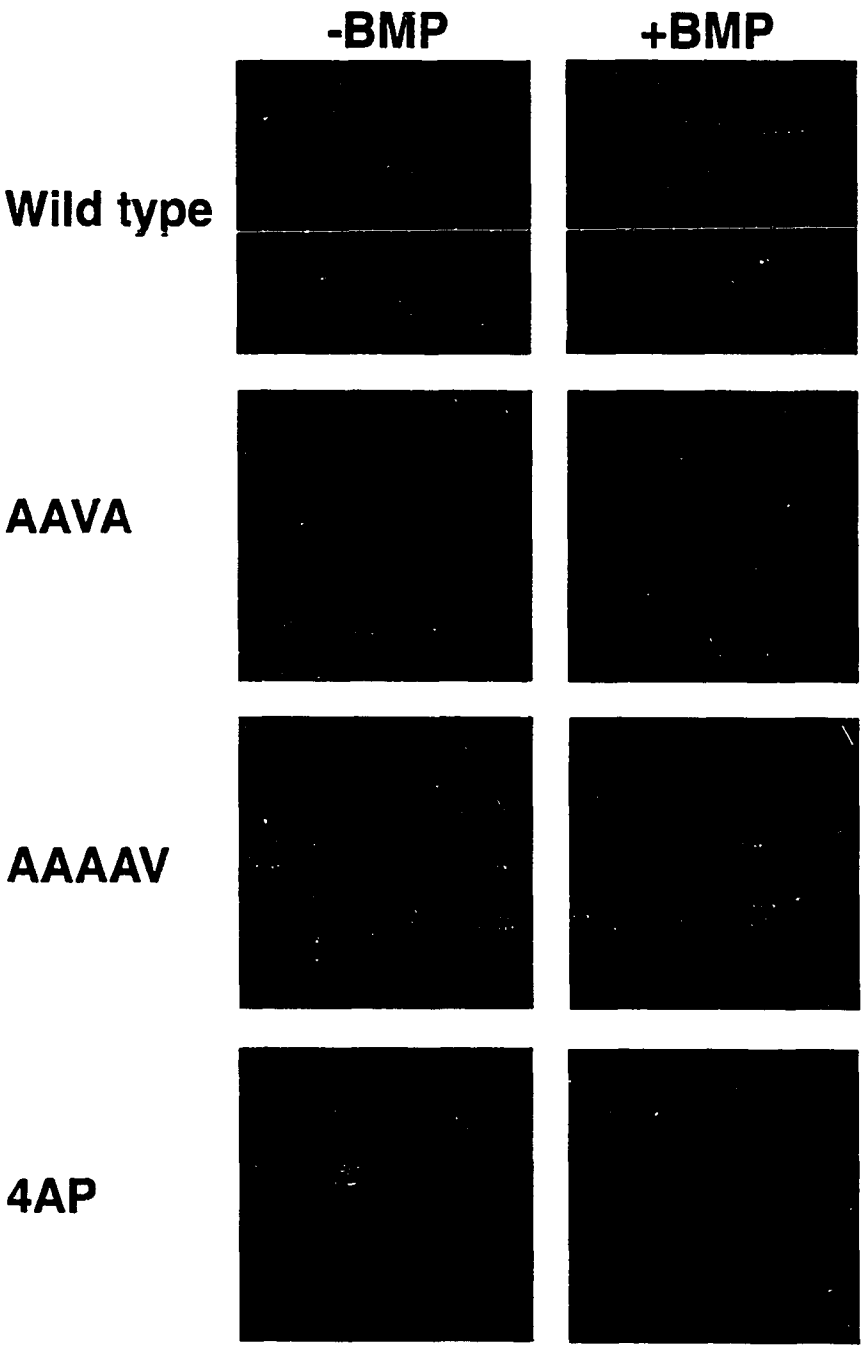

Figure 18. Nuclear localization of various Flag-Smad1 constructs in COS-1 cells. COS-1 cells were transfected transciently with Flag-tagged wild type Smad1 or mutant Smad1 (SSVS to AAVA in carboxyl-terminus, SSST to AAAV in linker region, and PXSP to PXAP (4AP) in the linker region). 5nMBMP2 was added for 30 min before immunostaining with M2 anti-Flag monoclonal antibodies and visualizing with FITC-conjugated secondary antibodies. Nuclei were visualized by counterstaining with DAPI.

	% nuclear	
Flag-Smad1	-BMP2	+BMP2
Wild-type	21	35
AAVA	8	9
AAAV	26	40
4AP	41	53

Figure 19. Nuclear localization of various Flag-Smad1 constructs. The Flag-Smad1 wild-type or mutant Flag-Smad1 constructs were transfected into COS-1 cells and visualized by immunoflourescence against the Flag epitope. 5nM BMP was added for 30 min before immunoflourescence where indicated. The percentage of cells with predominant nuclear staining is shown.

translocation. Transfected levels of Smad1 protein in COS-1 cells is high, which may account for some nuclear staining even in the absence of BMP stimulation (Fig.19). In the Smad 1(AAVA) mutant the amount of Smad1 in the nucleus is low whether or not BMP is added, suggesting that the SSVS motif is critical for nuclear translocation.

Whether nuclear translocation translates into transcriptional activation is determined by using Smad1 fused to the DNA-binding domain of GAL4 (Liu et al., 1996). This GAL4 fusion (with either Smad1 wild type or the Smad1(AAVA) mutant) was transfected into RIB/L17 cells together with a CAT reporter construct containing the GAL4 DNA-binding site. CAT activity measures transcriptional activation by Smad1. Smad1 fused to GAL4 has BMP2-inducible transcriptional activity that is inhibited significantly when the carboxy-terminal SSVS to AAVA mutation is introduced (Kretzschmar et al.,1997a). Therefore, loss of BMP-induced Smad1 phosphorylation correlates with failure of Smad1 to translocate to the nucleus and activate transcription. It is concluded that direct receptor phosphorylation of the carboxy-terminal serines of Smad1 is essential for functional activation of this protein.

On the other hand, mutations within the linker region have no effect on BMP-induced nuclear translocation (Fig.18). Both the AAVA and 4AP Smad1 mutants accumulate in the nucleus when cells are stimulated with BMP2, indicating that phosphorylation of the linker region (which is not BMP dependent) does not induce nuclear translocation. Indeed, the Smad1(4AP) mutant shows

elevated nuclear staining both in the presence and absence of BMP compared to wild-type. Smad1(4AP) in the absence of BMP has high levels of nuclear staining as well as cytoplasmic staining, contrary to wild type, which is predominantly cytoplasmic (Fig.18). This increase in nuclear staining is proportional to the number of PXSP sites mutated (Fig.19, data not shown) with maximal nuclear accumulation when all four PXSP sites are mutated. These results would suggest that phosphorylation of the PXSP motifs is not under the control of BMP. Mutation of the PXSP sites does not prevent BMP-induced nuclear translocation and may, in fact, be inhibitory of BMP stimulation. GAL4-Smad1(4AP) constructs transfected into RIB/L17 cells show a BMP-inducible increase in luciferase activity when co-transfected with a GAL4-dependent luciferase construct similar to wild-type Smad1 (Fig.20). Thus, the BMP-independent phosphorylation of the linker region does not induce nuclear localization nor does it stimulate BMP-responsive transcriptional activity.

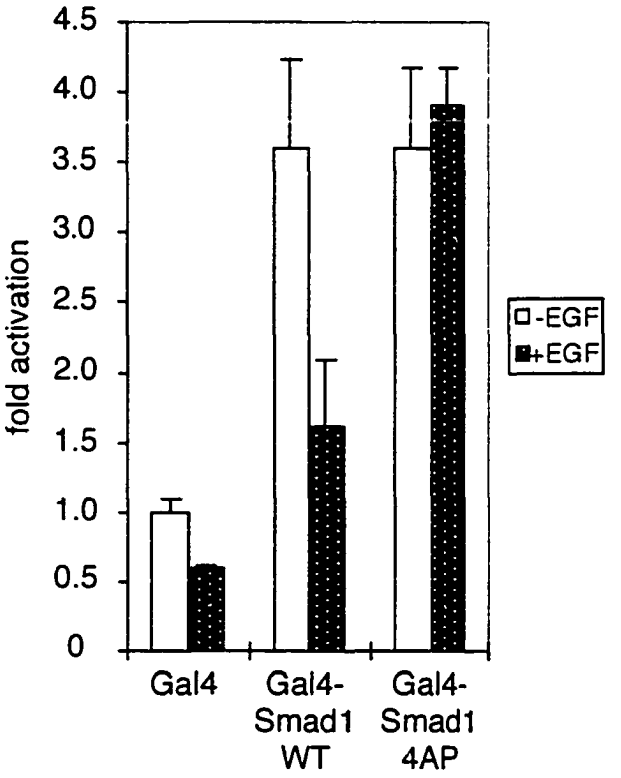

Figure 20. Phosphorylation in the linker region regulates transcriptional function of Smad1. R1B/L17 cells transfected with a Gal4-dependent luciferase reporter construct and the indicated Gal4 expression constructs were serum starved for 12 hours and then treated with or without BMP2 in the absence (white bars) or presence (black bars) of EGF. Luciferase activity was measured 18 hours after factor addition and plotted as relative activation by BMP2. Experiments were performed in triplicate.

Linker region phosphorylation of Smads by Erk2 in response to EGF

While constructing mutations of the serines in Smad and checking for nuclear translocation of these mutants I noticed an enhanced nuclear accumulation. These observations became the basis of two papers:

> Kretzschmar, M., Doody,J. and J.Massague (1997). Opposing BMP and EGF signalling pathways converge on the TGF-β family mediator Smad1. Nature, 389: 618-622.
>
> Kretzschmar, M., Doody,J., Timokhina,I. and J.Massague (1999). A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras. Genes Dev.,13: 804-816.

Dr. Marcus Kretzschmar focused on obtaining data showing effects of Smad phosphorylation by EGF which led to Figs. 22, 25, 26, 27, 28, 29, 30 and 32 where my role was to supply the appropriate mutants. I designed proteins to produce antibodies to Smad2 and Smad 3 (Fig. 34) necessary for visualization of Smad2 and Smad3 cellular localization (Fig. 35). The initial immunofluorescence that I obtained are shown in Figs. 33, 36 and 37 , the basis of which led to the continuation of this particular investigation. I also utilized the linker region protein construct I originally designed to ellicit antibodies to show phosphorylation of the linker *in vitro* (Fig. 31).

EGF increases Smad1 linker region phosphorylation

PXSP motifs are consensus sites for mitogen-activated protein kinases (MAPK) (Clark-Lewis et al., 1991; Gonzalez et al., 1991). Because basal phosphorylation of these sites in Smad1 is observed in cells in the presence of serum, the phosphorylation induced by specific growth factors known to activate MAP kinase was investigated. EGF signals through a receptor tyrosine kinase and strongly activates the Erk subfamily of MAP kinases (for review see Widmann et al., 1999) whereas tumor necrosis factor-α (TNFα) signals through a different family of receptors and stimulates the stress-activated MAP kinases Jnk and p38. To determine if Erk, Jnk or p38 is responsible for phosphorylation of Smad1, cells were transfected with Flag-Smad1 and stimulated with either EGF or TNF-α and immunoprecipitated 7 or 30 min after growth factor addition. EGF induces a rapid increase in Smad1 phosphorylation, whereas TNF-α has no effect (Fig.21). Mutating the four PXSP motifs abolished this EGF-induced phosphorylation as well as basal phosphorylation due to serum (Fig.22), indicating that growth factors such as EGF in the serum are responsible for phosphorylation of Smad1 at the PXSP sites. Phosphorylation by EGF is exclusive to the linker region because tryptic digestion of Smad1 reveals phosphorylation of the linker only while BMP2 shows phosphorylation both at the Carboxy-terminal tail and the linker region (Fig.23). Phosphorylation of the linker region in BMP2-treated cells is attributed to the effect of serum factors in the medium.

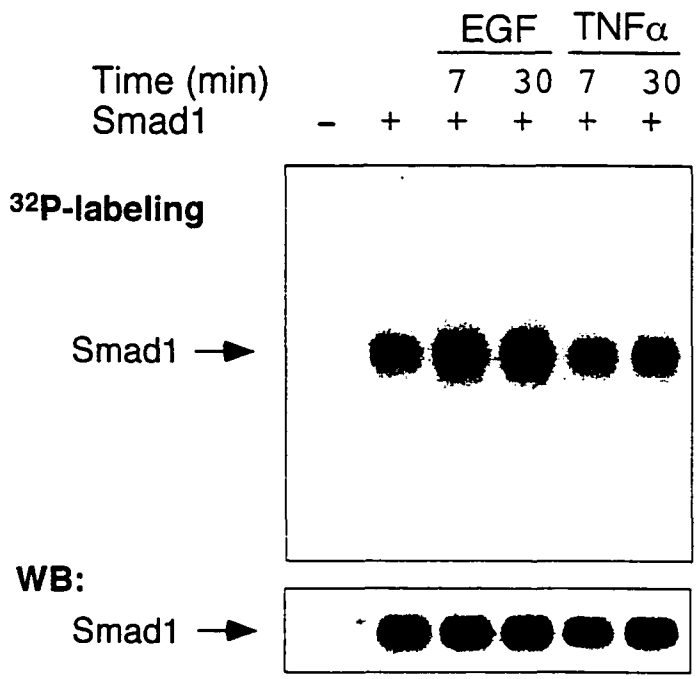

Figure 21. The effect of EGF and TNF-α on Smad1 phosphorylation in transfected R-1B/L17 cells. [32P]-labeled cells were incubated with either EGF or TNFa for the times indicated. Flag-Smad1 was immunoprecipitated and visualized by autoradiography or Western immunoblotting.

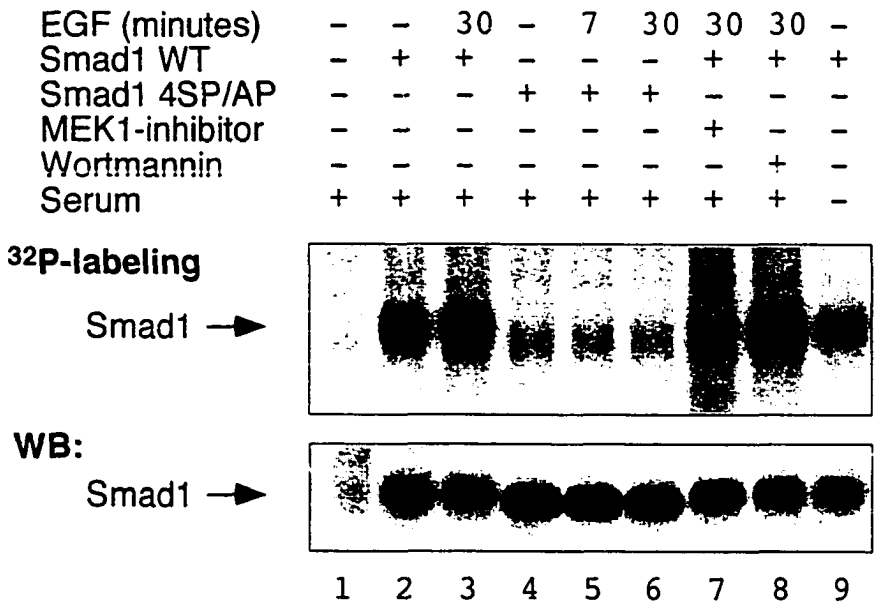

Figure 22. Effect of inhibitors and Smad1 PXSP site mutations on Smad1 phosphorylation in response to EGF. [^{32}P]-labeled cells were incubated with growth factor or inhibitors for the times indicated. Flag-Smad1 was immunoprecipitated with anti-Flag monclonal antibodies for either phosphate labeling or Western immunoblotting.

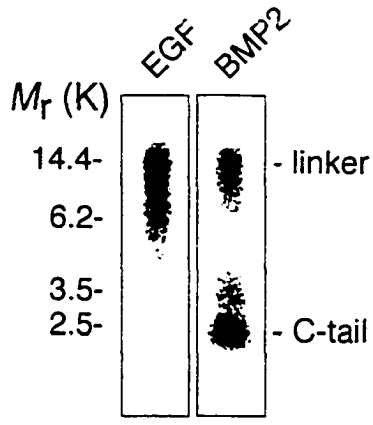

Figure 23. Tryptic digestion of Flag-Smad1 shows phosphorylation of linker region by EGF and carboxy-terminus by BMP. Trypsin digests of [^{32}P]-labeled Flag-Smad1 from EGF-treated or BMP2-treated cells . Phosphorylation of the linker in BMP2-treated cells is attributed to the effect of serum factors in the medium. Duplicate of Figure 14.

Two signaling pathways that mediate the effects of receptor tyrosine kinases are the Ras pathway, which leads to activation of Erk MAP kinases through Raf and MEK1 (Marshall,1995) and the phosphatidylinositol-3-OH kinase pathway, which activates the $p70^{S6K}$ and c-Akt kinases (Franke et al.,1997). Both basal phosphorylation and EGF-stimulated phosphorylation of Smad1 are strongly suppressed by a specific inhibitor of MEK1, PD98059 (Alessi et al.,1995), whereas wortmannin, an inhibitor of PI(3) kinase (Ui et al,1995), had no effect (Fig.22). Taken together, these results provide strong evidence that Smad1 is indeed a target of the Erk subfamily of MAP kinases in the cell.

EGF is responsible for linker region phosphorylation of Smad2 and Smad3

Smad2 and Smad3 which are activated by TGFβ and activin have high homology to Smad1. In the linker region of Smad2 and Smad3 are several PXS(T)P motifs, although fewer than found in Smad1 (Fig.24). Smad2 has only one PXS(T)P motif in the linker region while Smad 3 has two. However, the Smad2 linker region has three SP sequences and Smad3 has two. These sequences can serve as phosphorylation sites for proline-directed protein kinases including Erk (Davis,1993). The four S(T)P sites in both Smad2 and Smad3 were mutated to determine if these sites are necessary for EGF phosphorylation of Smad2 and Smad3 as they are for Smad1. When Flag-Smad2 or Flag-Smad3 constructs were transfected into RIB/L17 cells, immunoprecipitation of [^{32}P]labeled Smads showed that Smad2 and Smad3 are

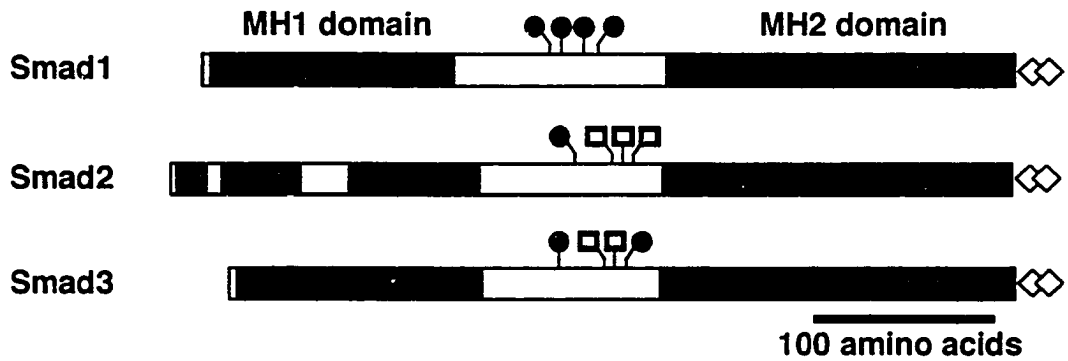

Figure 24. Schematic representation of phosphorylation sites.

(●) Erk concensus sites (PXS/TP) in the linker region that links the MH1 domain to the MH2 domain; (□) serine/proline motifs; (◇) receptor phosphorylation sites.

phosphorylated under normal culture conditions and the phosphorylation level of these proteins is increased by treatment with EGF (Fig.25). However, mutations of the 4S(T)P sites to A(V)P in both Smad2 and Smad3 (labeled 4AP for brevity) abolishes the phosphorylation of these mutants by EGF, showing no increase over basal levels. Therefore, EGF phosphorylates Smad2 and Smad3 at the S(T)/P sites corresponding to those of Smad1.

Whether, as in Smad1, Erk is responsible for the phosphorylation of Smad2 and Smad3 by EGF stimulation is determined by incubating RIB/L17 cells with either MEK1 inhibitor or wortmannin. As in Smad1, phosphorylation of the S/TP sites of Smad2 and Smad3 decreases when inhibiting with MEK1. However, unlike Smad1, wortmannin also inhibits Smad2 and Smad3 phosphorylation, albeit to a lesser extent (Figs.26,27). Cotransfection of activated Ras or MEK1 increases the phosphorylation of Smad2 and Smad3 implicating Ras and MEK1 in Smad phosphorylation. Compared with the transfected Smad2 and Smad3, their corresponding 4AP constructs show very low levels of phosphorylation and these levels are not increased by H-Ras^{V12} (Figs.26,27). That EGF and Ras induces Smad2 and Smad3 phosphorylation in the linker region but not the C-terminal tail (which is the site of TGFβ activation) is evident when using carboxy-terminal mutant Smad2 and Smad3 constructs. When Flag-tagged Smad constructs containing serine to alanine mutations in the Carboxy-terminal SSXS are cotransfected with H-Ras^{V12} into RIB/L17 cells phosphorylation levels become elevated (Fig.28). The same is true when AAXA

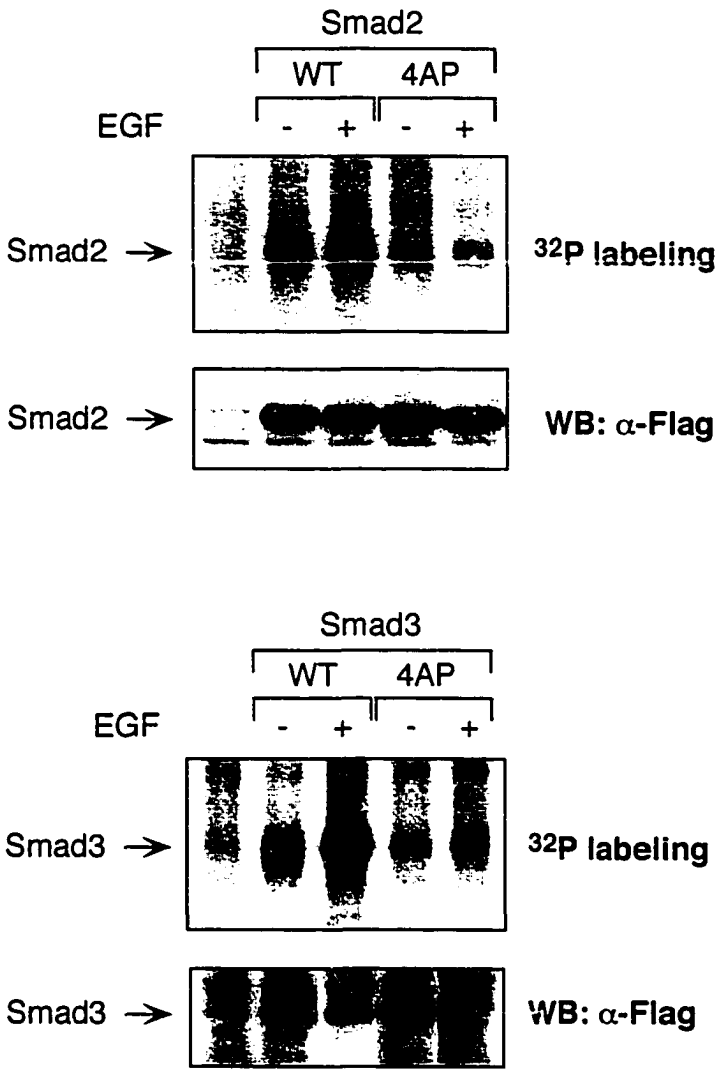

Figure 25. EGF induces phosphorylation of Smad2 and Smad3.

R1B/L17 cells transfected with vectors encoding the indicated Flag-tagged Smad constructs were labeled with ^{32}P, and incubated with EGF for 30 min. The phosphorylation level of the transfected Smads was determined by anti-Flag immunoprecipitation. Smad expression was controlled by anti-Flag immunoblotting of cell lysates.

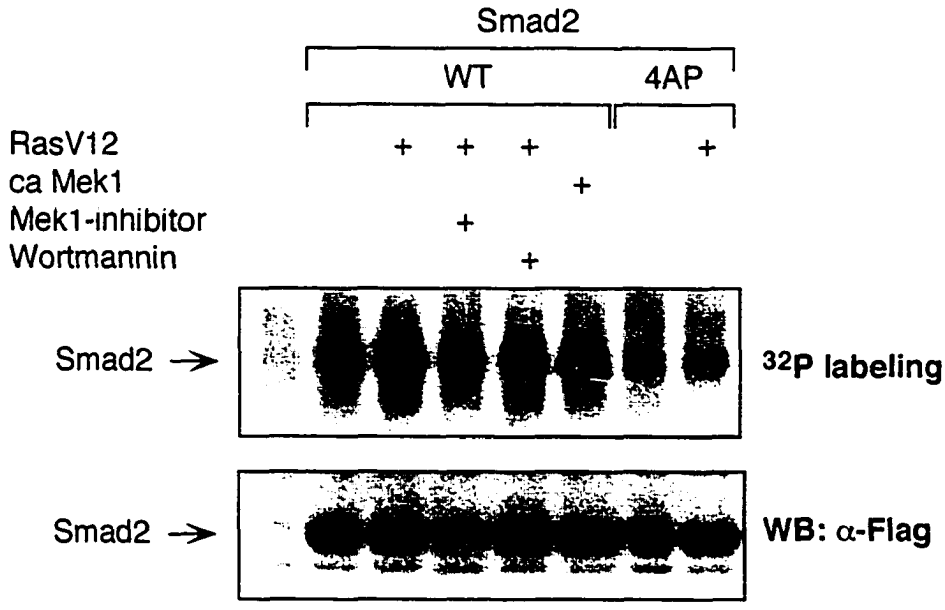

Figure 26. Activated Ras and Mek1 causes phosphorylation of Smad2. Vectors encoding Flag-tagged wild-type (WT) Smad2 or mutant Smad2 lacking all four potential phosphorylation sites in the linker region (4AP) were used. These constructs were cotransfected with vectors encoding H-Ras^{V12} or constitutively active Mek1 (caMek1) into R1B/L17 cells as indicated. The phosphorylation level of transfected Smad2 was determined by anti-Flag immunoprecipitation from ^{32}P-labeled cells. Mek1 inhibitor or wortmannin were added 1.5hr prior to cell lysis, as indicated. Smad expression was monitored by anti-Flag immunoblotting of cell lysates.

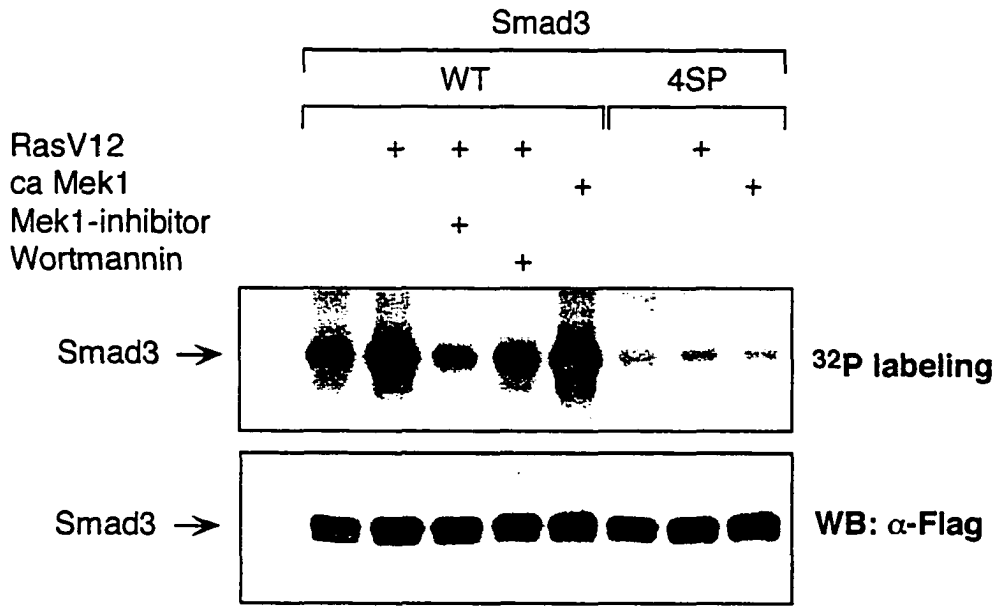

Figure 27. Activated Ras and Mek1 causes phosphorylation of Smad3. Vectors encoding Flag-tagged wild-type (WT) Smad3 or mutant Smad3 lacking all four potential phosphorylation sites in the linker region (4AP) were used. These constructs were cotransfected with vectors encoding H-Ras^{V12} or constitutively active Mek1 into R1B/L17 cells as indicated. The phosphorylation level of transfected Smad3 was determined by anti-Flag immunoprecipitation from ^{32}P-labeled cells. Mek1 inhibitor or wortmannin were added 1.5hr prior to cell lysis, as indicated. Smad expression was monitored by anti-Flag immunoblotting of cell lysates.

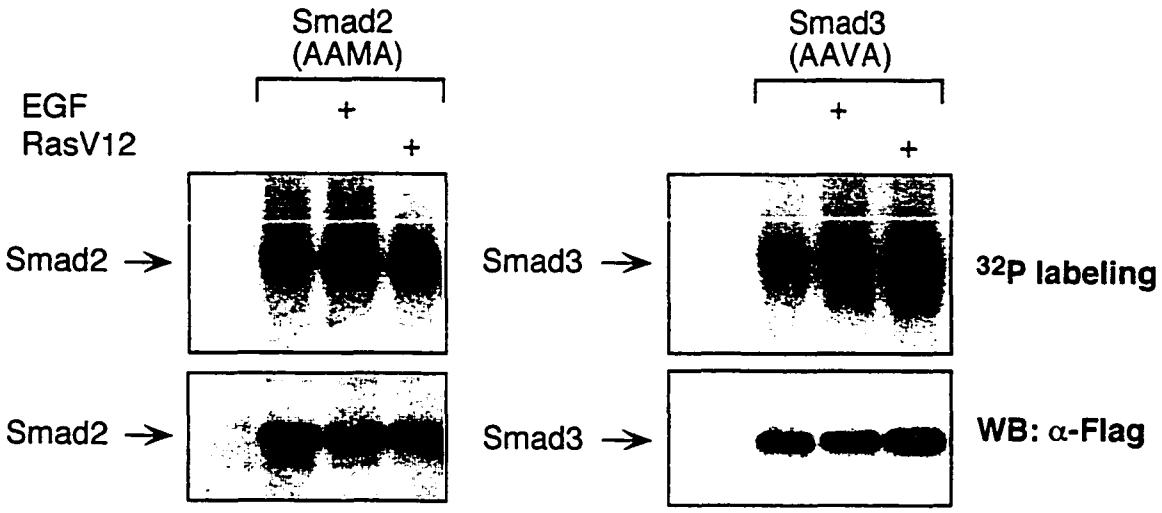

Figure 28. Phosphorylation of Smad2 and Smad3 by EGF or Ras^{V12} is not to the SSXS motif on the carboxy-terminal tail. Effects of H-Ras^{V12} cotransfection or EGF addition (18nM, 30min) on the phosphorylation of Flag-tagged Smad constructs containing S to A mutations in the carboxy-terminal SSXS receptor phosphorylation sites. Smad expression was monitored by anti-Flag immunoblottiing of cell lysates.

mutants are stimulated with EGF. Thus, phosphorylation of Smad2 and Smad3 is undiminished, arguing that the TGFβ receptor type 1 phosphorylation sites are not required for EGF-induced phosphorylation. In conclusion, Smad2 and Smad3 are phosphorylated at the S/TP sites of the linker region by EGF stimulation of Erk and Ras.

Erk2 phosphorylates Smad at SP sites in vitro

To provide evidence that Erk kinases can phosphorylate Smads directly at the identified sites, bacterially expressed, purified Smads and mutant Smads(4AP) were used as substrates in *in vitro* kinase assays. Both Smad1 and Smad1(4AP) are equally well phosphorylated when titrated in kinase reactions with constitutively activated BMPRI-IB (Fig.29). As indicated earlier, the BMP receptor kinase phosphorylates Smad1 at the C-terminal serines and the linker mutations do not affect BMP-induced phosphorylation of Smad1 *in vivo* (Figs.16,17). Titration of wild-type Smad1 in kinase reactions with activated Erk2 demonstrate that wild-type Smad1 is a substrate for Erk2 at nanomolar concentrations *in vitro* (Fig.30). In contrast to the BMP receptor kinase, Erk2 fails to phosphorylate Smad1(4AP). Moreover, the isolated recombinant linker region of Smad1 is also phosphorylated by Erk2 (Fig.31). Thus Erk2 can phosphorylate Smad1 *in vitro* at the same sites that are phosphorylated in response to EGF *in vivo* and the linker region is the only area phosphorylated by Erk2. When purified recombinant Smad2 and Smad3 are used as substrates in *in vitro* kinase assays

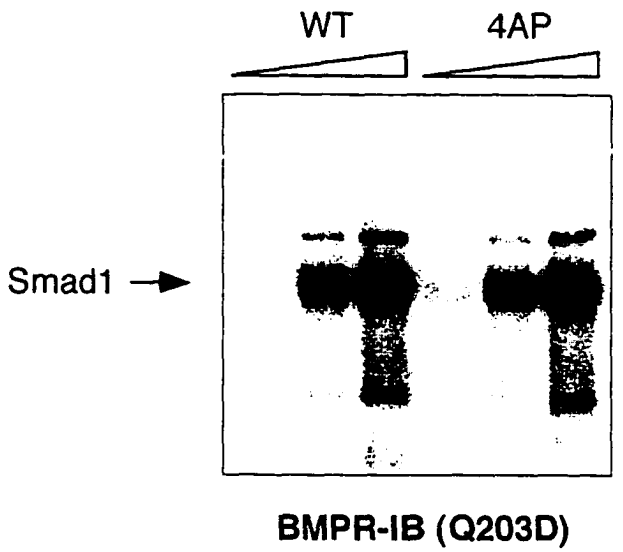

Figure 29. Smad1 is phosphorylated by BMP receptor type1 at a site other than the linker region. Purified, recombinant Smad1 proteins (wild-type(WT) or the quadruple PXSP mutant (4AP)) were tested at concentrations in the nanomolar range as substrates of recombinant activated BMP type I receptor kinase (BMPR-IB (Q203D)). Lanes 1 and two are a duplicate of Figure 12.

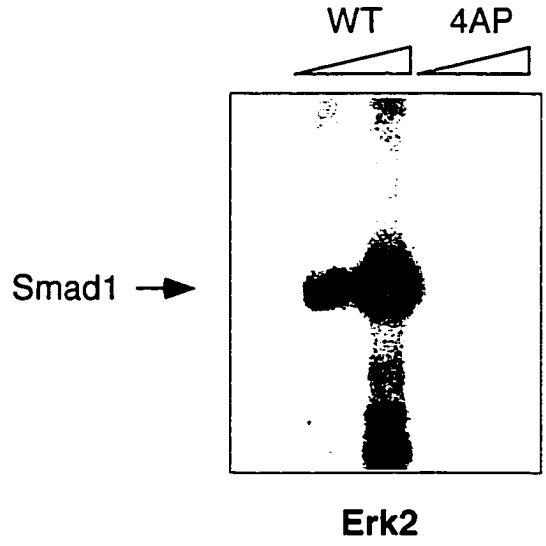

Figure 30. Smad1 is phosphorylated by Erk2 at PXSP sites *in vitro.*

Purified, recombinant wild-type Smad1 or the Smad1(4AP) mutant were tested at concentrations in the nanomolar range as substrates of recombinant activated Erk2.

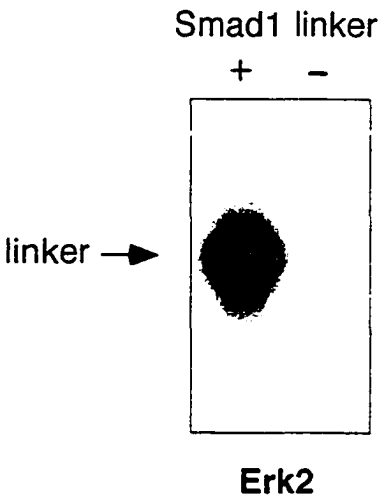

Figure 31. Smad1 is phosphorylated by Erk2 in the linker domain. A purified, recombinant Smad1 linker domain containing the 4SP sites was tested in a kinase assay with purified activated Erk2.

with Erk2 similar results as with Smad1 are shown (Fig32). Erk phosphorylates both Smad2 and Smad3 *in vitro* and this phosphorylation is predominantly at the 4S(T)P sited located in the linker region of these proteins.

Phosphorylation of the linker region of Smad regulates cellular localization

To understand the functional significance of Smad phosphorylation by MAP kinases, the potential consequences on Smad localization within the cell are investigated. Both addition of EGF and the mutations of the SP sites have striking effects on the subcellular localization of Smad1. Immunofluorescence of Smad1-transfected cells reveals a distribution throughout the cell under basal conditions and a predominantly nuclear localization after BMP2 stimulation (Figs.18,33). The mutant Smad1 (4AP) is predominantly nuclear even in the absence of BMP stimulation (Fig.33) suggesting that phosphorylation of the 4SP sites is inhibitory. Indeed, treating cells with EGF causes exclusion of wild-type Smad1 from the nucleus and inhibits BMP-induced nuclear accumulation from these same cells but has no effect on the mutant Smad1. Furthermore, neither addition of MEK1 inhibitor nor serum starvation lead to nuclear accumulation even in the absence of treatment with BMP. MEK1 inhibitor or serum starvation have no effect on the mutant Smad1. These results suggest the Erk-mediated phosphorylation of the linker region inhibits nuclear accumulation induced normally by BMP.

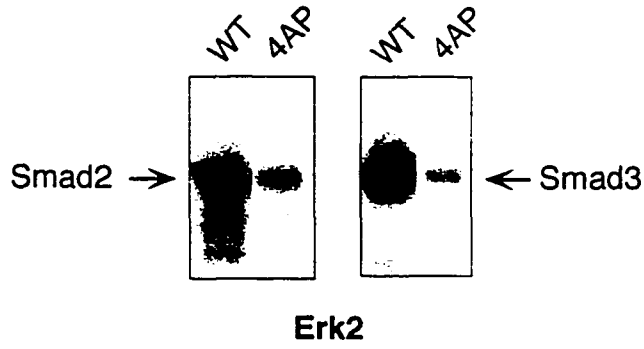

Figure 32. Erk2 causes phosphorylation of Smad2 and Smad3 *in vitro*. Purified, recombinant Smad proteins (wild-type or 4AP mutants) were tested as substrates for recombinant activated Erk2 in *in vitro* kinase assays.

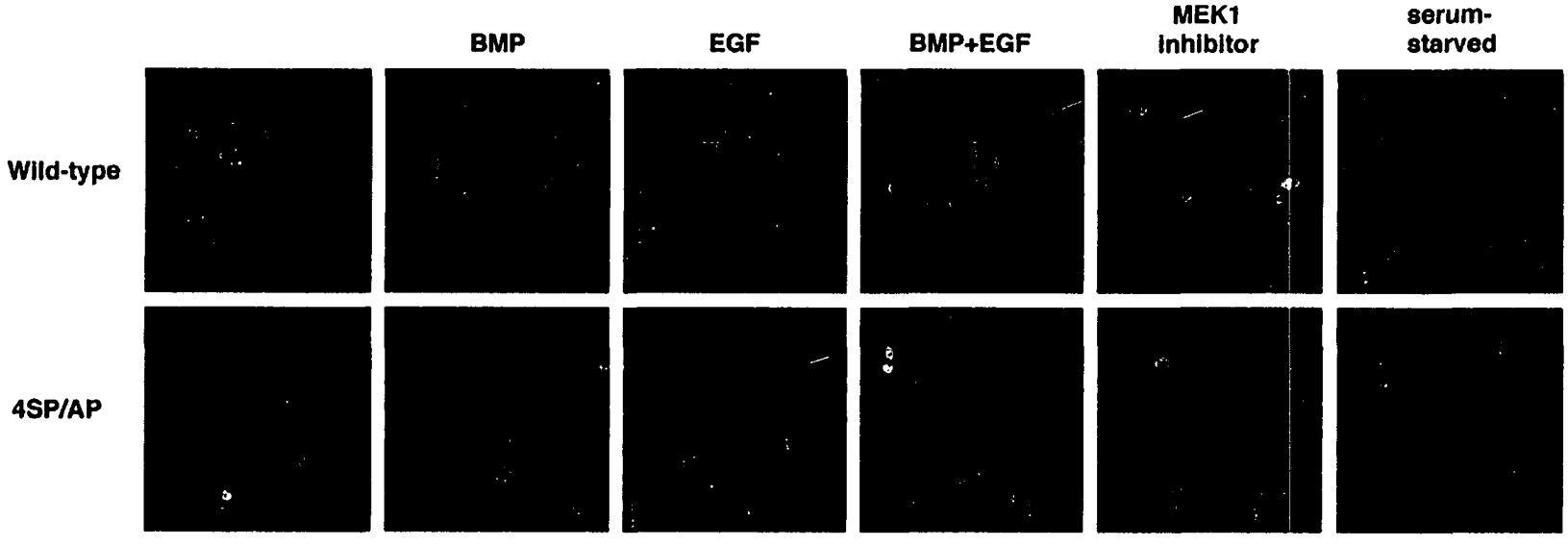

Figure 33. Phosphorylation in the linker region regulates cellular localization of Smad1. Cos1 cells were transiently transfected with Flag-tagged wild-type Smad1 or the mutant Smad1(4AP). Where indicated, cells were treated with EGF 15 minutes before BMP2 addition. Then, 30 minutes after BMP2 addition, immunostaining was performed using anti-Flag monoclonal antibody and FITC-conjugated secondary antibody. Treatment with MEK1 inhibitor and serum starvation was for 1 hour and for 16 hours respectively, before immunostaining. These same slides were counterstained with DAPI to visualize nuclei (data not shown).

Polyclonal antibodies raised against the linker region of Smad1 are used in immunofluorescence to verify if endogenous Smad1 mimics transfected protein in relation to EGF. When BMP is added to cells, endogenous Smad1 translocates from the cytoplasm to the nucleus, comparable to transfected Smad1 protein (data not shown). However, this translocation is inhibited when cells are treated with EGF indicating the results with transfected Smad1 correlate with those of endogenous Smad1, where EGF inhibits BMP-induced nuclear accumulation.

TGFβ-induced nuclear localization of Smad2 and Smad3 are also affected by EGF. Rabbit polyclonal antibodies were raised against the recombinant linker region of Smad2 to see if endogenous levels of Smad2 in the nucleus would be affected by EGF treatment. These antibodies recognize Smad2 and, to a lesser extent Smad3, but not Smad1 or Smad4 (Fig.34). This crossreaction of the antibody to Smad3 is consistent with the high homology between these two proteins (even in the linker region) and I will designate this antibody as an anti-Smad2/3 antibody. To investigate whether Smad2 and Smad3 duplicated the results seen with Smad1, Mv1Lu cells were subjected to TGFβ treatment and cells were visualized by immunofluorescence with the anti-Smad2/3 antibody. Endogenous Smads show generalized staining throughout the cell when no growth factors are added, similar to what is seen with Smad1 (Fig.35). Upon TGFβ addition, almost all cells in the population show intense nuclear accumulation of Smad2/3. When TGFβ is given in combination with EGF

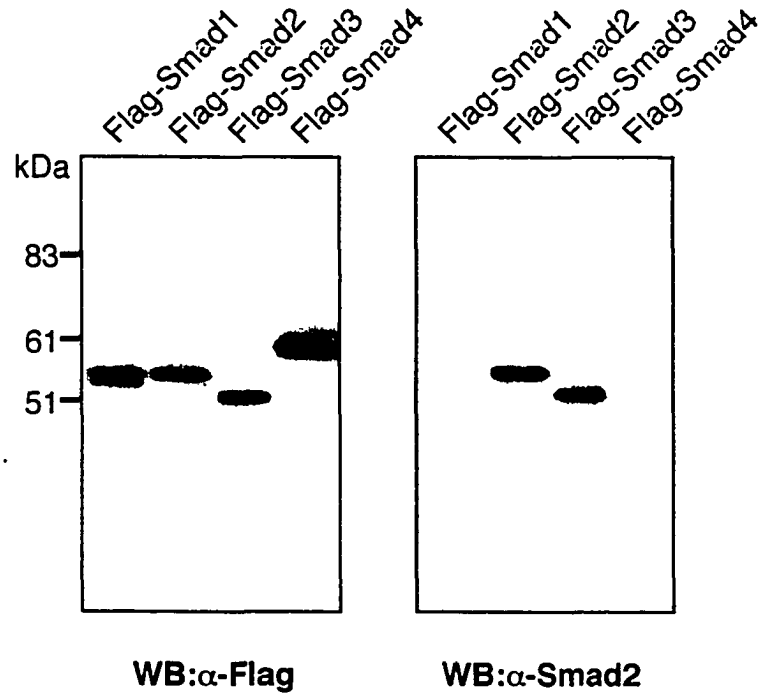

Figure 34. Specificity of antibodies generated against Smad2.

COS-1 cells transfected with Flag-tagged versions of Smad1, Smad2, Smad3 or Smad4 were subjected to Western immunoblotting with anti-Flag antibodies as a positive control, or antibodies raised against the linker region of Smad2.

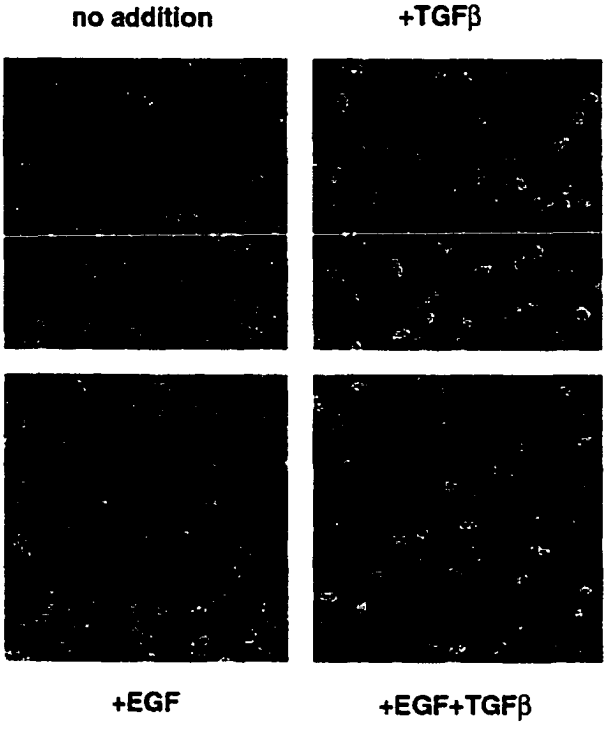

Figure 35. EGF inhibits TGFβ-dependent nuclear accumulation of Smad2 and Smad3. Mv1Lu cells were incubated with EGF for 60min before fixation (bottom) and/or TGFβ for 30min before fixation (right), and endogenous Smad2/3 visualized by immunoflourescence. Note the lack of cytoplasmic staining in cells treated with TGFβ alone, and the cytoplasmic staining remaining in cells treated with TGFβ plus EGF.

however, a substantial level of Smad2/3 staining remains in the cytoplasm in most cells. EGF inhibition is more effective at preventing nuclear translocation of Smad2/3 as TGFβ levels are decreased, suggesting that the extent of Smad2/3 nuclear accumulation depends on the relative strengths of counterbalancing signals (data not shown). Therefore, as in Smad1, Smad2 and Smad3 is inhibited from accumulation in the nucleus by treatment with EGF.

The inhibition of Smad2/3 nuclear translocation with EGF equally requires phosphorylation in the linker domain analogous to that seen with Smad1 inhibition. EGF and its signaling partners, MEK1 and Ras, are responsible for phosphorylating Smad2 and Smad3 in the linker region. When Mv1Lu cells are cotransfected with wild-type Smad3 and activated MEK1, Smad1 remains cytoplasmic in the cell, even when stimulated with TGFβ (Figs.36,37). Removal of the 4S(T)P sites in Smad3 abolishes this inhibition and cells become refractory to the effects of MEK1. Indeed, even cells with no TGFβ treatment show some nuclear staining of Smad3. Inhibition of Smad3 nuclear accumulation by EGF and activated MEK1 therefore requires the linker region phosphorylation sites, mimicking the results seen with Smad1.

To assess the functional consequences of Smad1 regulation by EGF, the effects of EGF treatment on the transcriptional activity of Smad1 in response to BMP were analyzed. Since no mammalian response genes are available for assaying BMP activity, a GAL4-Smad1 fusion protein is used to demonstrate BMP activation of transcriptional responses. When GAL4-Smad1 wild type is

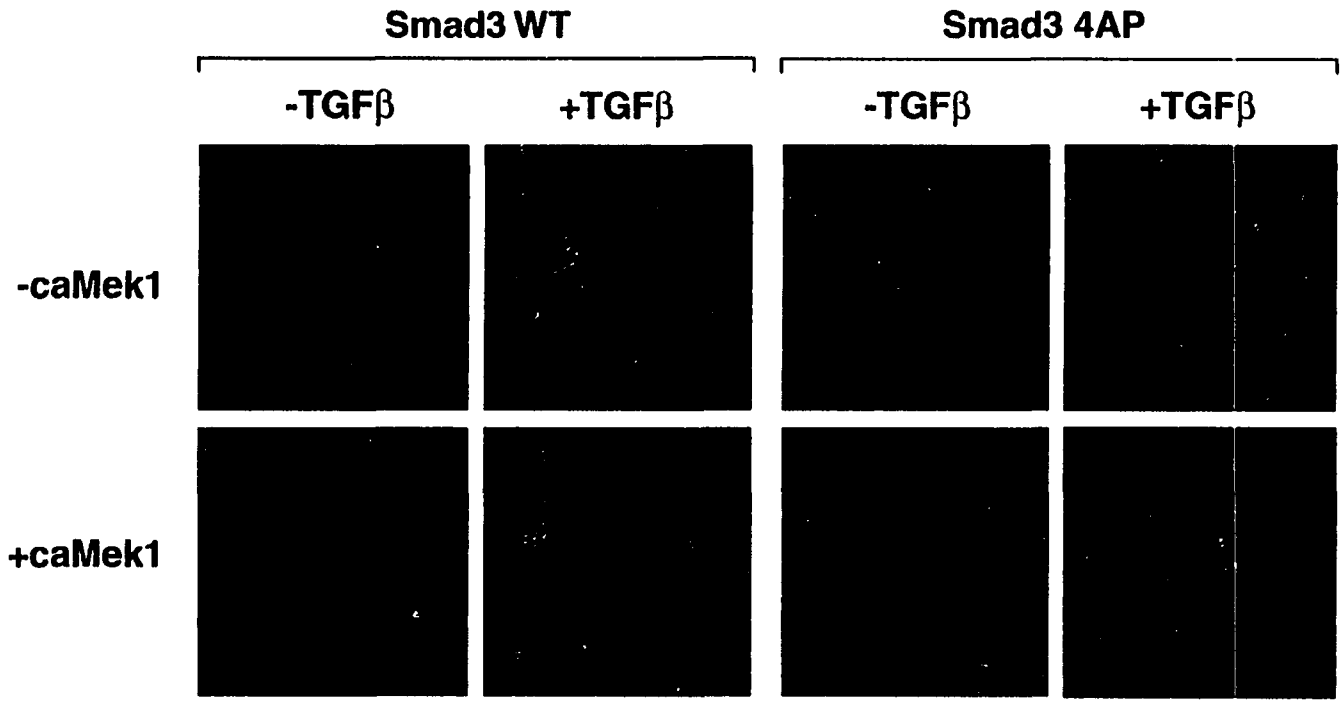

Figure 36. Mek1 inhibition of Smad3 nuclear accumulation requires phosphorylation in the linker region. Mv1Lu cells were cotransfected with Flag-tagged Smad3 (WT) or mutant (4AP) and empty vector or constitutively active Mek1 as indicated. TGFβ was added 30 min prior to fixation. Flag-Smad3 was visualized by anti-Flag immunofluorescence.

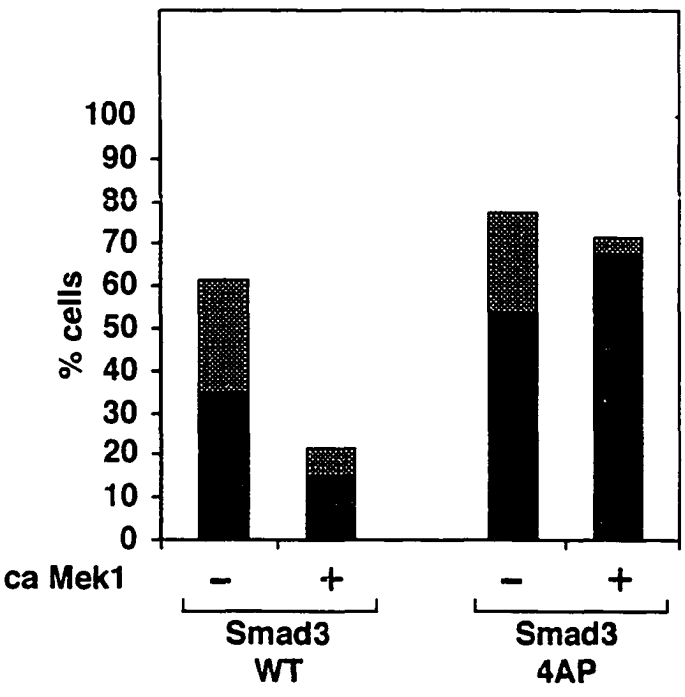

Figure 37. Mek1 inhibition of Smad3 nuclear accumulation requires phosphorylation in the linker domain. Nuclear accumulation of Flag-Smad3 in the TGFβ-treated cells of Figure 36 was quantitated by determining the proportion of cells with Smad2/3 staining exclusively in the nucleus (solid area) and cells with predominant but not exclusive nuclear staining (stippled area).

transfected into RIB/L17 cells, there is a larger that 3-fold increase in luciferase activity (Fig.20). The GAL4-Smad1(4AP) mutant construct is induced by BMP to the same extent as the wild-type construct. Most importantly, simultaneous treatment of cells with EGF reduces the BMP-induced transcriptional activity of GAL4-Smad1, whereas EGF has no effect on the mutant Smad1. This suggests that Erk-mediated phosphorylation of Smad1 inhibits BMP-induced nuclear accumulation and consequently inhibits nuclear functions of Smad1, such as transcriptional activity.

Oncogenic Ras represses TGFβ signaling

Results presented in this section were incorporated with the previous section in:

> Kretzschmar, M., Doody,J., Timokhina,I. and J.Massague (1999). A mechanism of repression of TGFβ/Smad signaling by oncogenic Ras. Genes Dev.,13: 804-816.

I performed all experiments shown except for those referred to in Figs. 45 and 46. Since nuclear translocation proved to be a reliable indicator of Smad function, I was able to use this technique to prove the inhibitory effects of EGF, Mek1 and Ras on TGFβ signaling. Moreover, I had produced antibodies which allowed the visualization of endogenous Smads to show these results were identical to those seen with overexpression of a Smad construct. These antibodies also allowed me to assess that in cells overexpressing an oncogenic Ras, the TGFβ signaling pathway remained intact.

Ras induces a higher phosphorylation of Smad than EGF

Constitutively active oncogenic Ras, or its downstream partners, MEK1, increases Smad2 and Smad3 phosphorylation (Figs.26,27) *in vivo*, indicating that Ras activation by EGF is one mechanism by which Smad transcriptional activity is inhibited. In many instances, phosphorylation of Smad2 and Smad3 by oncogenic Ras or constitutively activated MEK1 is more intense than with EGF (Fig.28, data not shown). This increase in phosphorylation of Smad2 and Smad3 by oncogenic Ras correlates with Ras effect on nuclear accumulation. Oncogenic Ras dramatically inhibits nuclear accumulation in transiently transfected Mv1Lu cells (Figs.38,39). Cotransfection of green fluorescence protein (GFP) serves to distinguish transfected from nontransfected cells. Where almost all the cells show nuclear accumulation of endogenous Smad2/3 in response to TGFβ, only a small proportion of H-Ras^{V12} or activated MEK1 transfected cells show nuclear accumulation. GFP-negative cells, which serve as internal controls in these assays, have Smad2/3 staining in the nucleus in the presence of TGFβ. Therefore H-Ras^{V12} is a potent regulator of Smad nuclear translocation.

Oncogenic Ras affects TGFβ signaling

Since oncogenic Ras affects Smad nuclear accumulation, it should also affect signaling by TGFβ. To be able to dissect oncogenic Ras effects on the TGFβ signaling pathway a well characterized mouse mammary epithelial cell

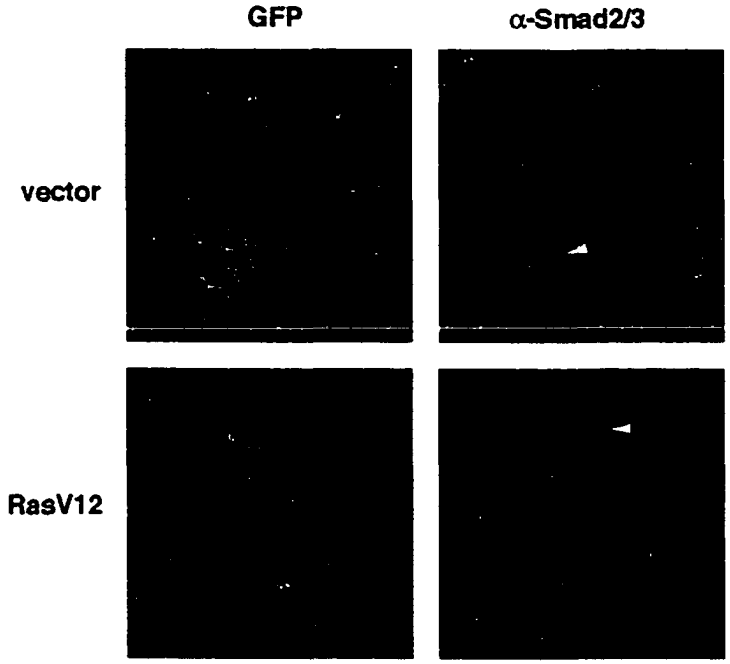

Figure 38. Nuclear accumulation of Smads in response to TGFβ is inhibited by oncogenic H-Ras^{V12} or constitutively active Mek1. MviLu cells were transiently transfected with empty vector, H-Ras^{V12} or constitutively active Mek1 (ca Mek1), and then treated with TGFβ for 30 min before fixation. Transfected cells were marked by cotransfection with GFP. Endogenous proteins were visualized by anti-Smad2/3 immunofluorescence by a rhodamine-coupled system. (Arrowheads) GFP-positive cells.

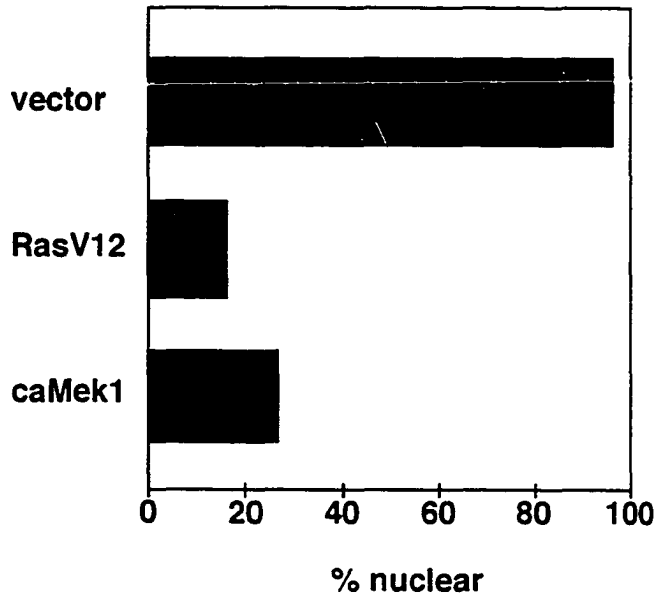

Figure 39. Nuclear accumulation of Smads in response to TGFβ is inhibited by oncogenic H-Ras^{V12} or constitutively active Mek1. Quantitation of Figure 38. Percentage of GFP-positive cells with Smad2/3 predominantly in the nucleus under the three experimental conditions.

system was used (Oft et al.,1996). The parental cell line EpH4 is nontumorigenic and responds to TGFβ in various assays. A $^{v\text{-}12}$Ha-Ras transformed derivative, EpRas, not growth inhibited by TGFβ, transforms into a fibroblastoid phenotype and is highly invasive *in vivo* (Oft et al.,1996). To investigate whether EpRas cells accumulate Ras-independent defects in TGFβ signaling over time, Smad2 and Smad3 activation in both EpH4 and EpRas are verified (Fig.40). Levels of both Smad2 and Smad3 protein are similar between EpH4 and EpRas, and are comparable to levels seen in Mv1Lu cells which have a good TGFβ response. Using antibodies specifically recognizing TGFβ receptor-phosphorylated Smad2, it is determined that endogenous Smad2 in both EpH4 and EpRas cells is phosphorylated in response to TGFβ (Fig.41). Thus, Ras transformation does not interfere with TGFβ receptor-mediated phosphorylation of Smad2 in EpRas cells.

Next, the phosphorylation status of Smad in both EpH4 and EpRas cell lines was investigated. Activated Ras propagates signals through the activation of Erk2 among other protein kinases (Davis,1993). The level of Erk2 is similar in EpH4 and EpRas cells, as determined by Western immunoblotting with anti-Erk antibodies (Kretzschmar et al., 1999). However, the level of Erk activity is higher in EpRas cells than in EpH4 cells, as determined by measuring the kinase activity of Erk immunoprecipitates (Kretzschmar et al., 1999). This result is consistent with the presence of hyperactive Ras in EpRas cells. Similarly, immunoprecipitation of endogenous Smad2 and Smad3 shows that the level of

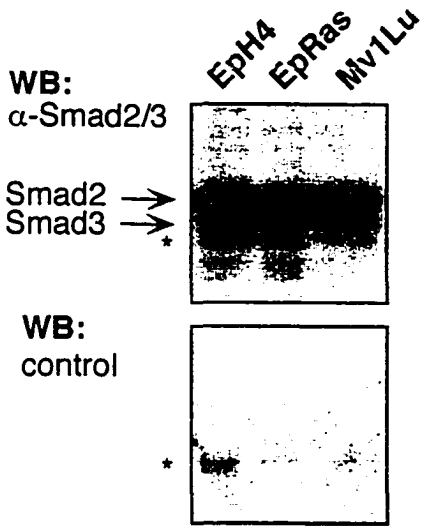

Figure 40. Smad2 and Smad3 expression in EpH4, EpRas and Mv1Lu cells. Untransfected EpH4, EpRas and Mv1Lu cell extracts were subjected to Western immunoblotting with affinity-purified anti-Smad2/Smad3 (top) or non-immune serum (bottom). (Asterisks)Non-specific bands.

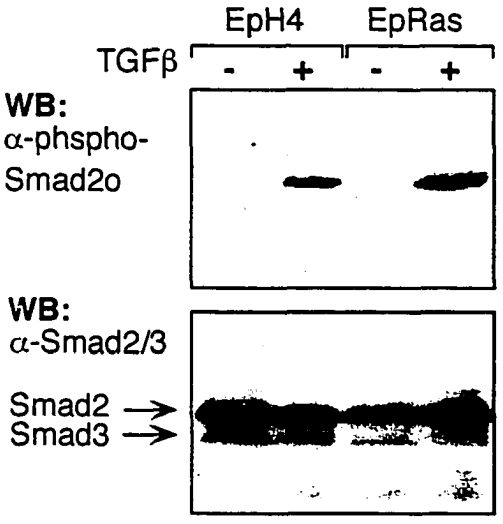

Figure 41. TGFβ–induced phosphorylation of Smad2 in EpH4 and EpRas cells. EpH4 and EpRas cells were stimulated with TGFβ for 30 min. Cell lysates were subjected to Western immunoblotting with either antibodies agains receptor-phosphorylated Smad2 (top) or anti-Smad2/3 antibodies (bottom).

phosphorylation of these proteins is higher in EpRas cells than in EpH4 cells (Fig.42). Elution of the [^{32}P] labeled EpRas Smad band from these gels, followed by trypsin digestion, yields a product of 13kD, indicating the linker region is phosphorylated. Smad2 and Smad3 are therefore phosphorylated in response to oncogenic Ras at sites situated in the linker region of these proteins, reminiscent of the results seen with EGF, activated MEK1 and activated Ras in Mv1Lu cells.

Immunofluorescence patterns of endogenous Smad2 and Smad3 protein in EpH4 and EpRas cells was determined using anti-Smad2/3 antibodies. In the absence of TGFβ, both cell lines show Smad2/3 staining throughout the cell. Upon TGFβ addition, greater than 95% of the EpH4 cells show an accumulation of Smad2/3 immunostaining in the nucleus as compared to 10% in EpRas cells (Fig.43). This accumulation of Smad2/3 in EpH4 cells is rapid and was sustained for over three hours (Fig44). In contrast, EpRas cells respond to TGFβ with a limited accumulation of Smad2/3 in the nucleus. Furthermore, this accumulation is slow and only partial, as most cells with predominant nuclear staining still show some staining of the cytoplasm. Therefore, cells which contain oncogenic Ras partially inhibit TGFβ-induced nuclear accumulation of Smad2 and Smad3. Increasing amounts of TGFβ incrementally increase TGFβ-induced nuclear translocation in EpRas cells with high doses (100pmol or more) overcoming Ras effects on Smad2 and Smad3 nuclear translocation (data not shown).

To investigate Ras as an antagonist of TGFβ signaling we used a Smad target promoter for the *Mix.2* gene from *Xenopus* was used (Chen et al.,1996;

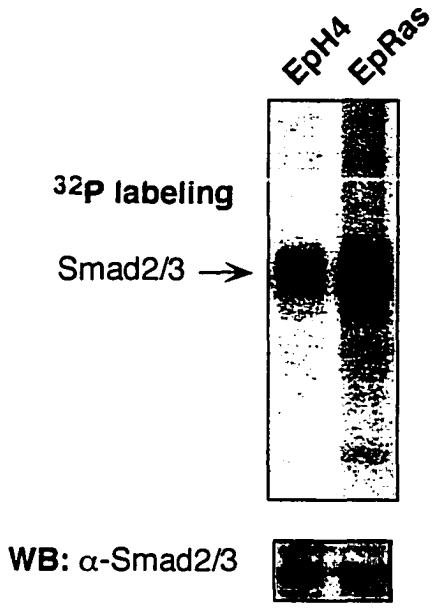

Figure 42. Elevated Smad2/3 phosphorylation levels in EpRas cells.
The phosphorylation level of Smad2 and Smad3 in EpH4 and EpRas cells was determined by immunoprecipitation from ^{32}P-labeled cells. The relative levels of Smads were determined by anti-Smad2/3 Western immunoblotting of unlabeled cell extracts prepared in parallel.

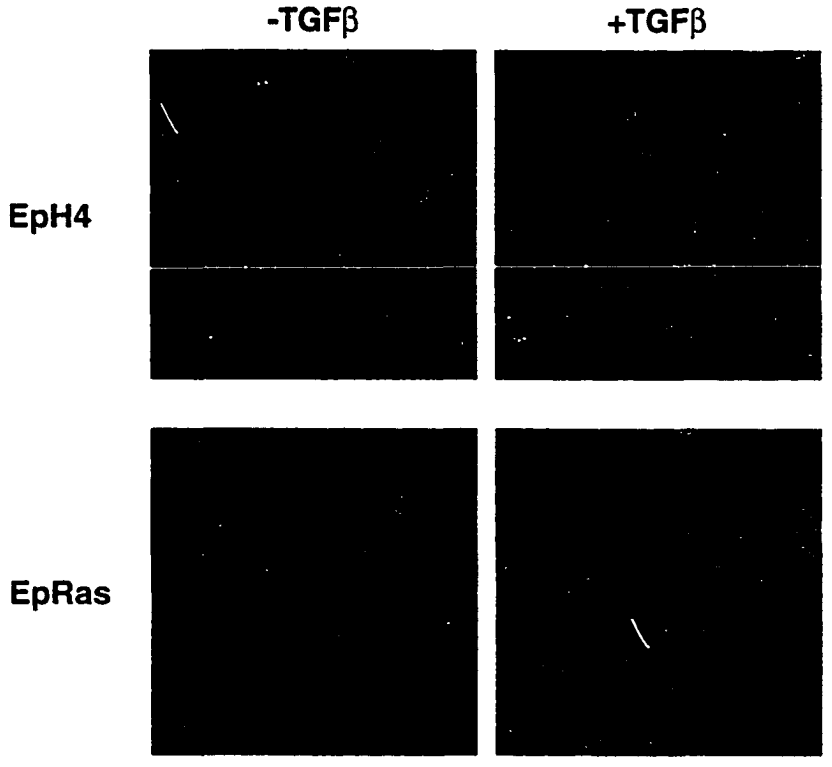

Figure 43. TGFβ-induced nuclear accumulation of Smad2/3 in EpH4 and EpRas. EpRas and EpH4 cells were stimulated with TGFβ for 30 min. Endogenous Smad2 and Smad3 were visualized by anti-Smad2/3 immunofluorescence.

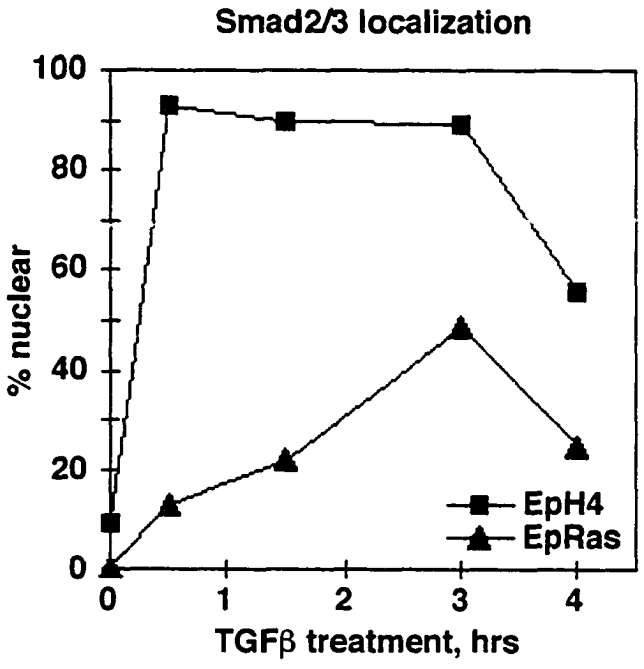

Figure 44. TGFβ-induced nuclear accumulation of Smad2/3 is temporally decreased in EpRas cells. EpH4 (▲) and EpRas (■) cells were treated with TGFβ for different time periods. Following immunofluorescence staining, the percentage of cells with Smad2/3 staining predominantly or exclusively in the nucleus was determined.

Chen et al.,1997b; Liu et al.,1997). On translocation into the nucleus, receptor-activated Smad2 or Smad3 associates with the DNA-binding protein FAST2 to form a transcriptional complex on the activin/TGFβ response element (ARE) of the *Mix.2* promoter (Labbe et al.,1998; Liu et al., 1999). Strong activation of an ARE reporter construct (A3-Luc) by TGFβ is achieved in EpH4 cells cotransfected with FAST2 (Fig.45, Kretzschmar et al., 1999). In contrast, FAST2-transfected EpRas cells show only a weak activation of A3-Luc in response to TGFβ, suggesting that Smad signaling is impaired. When Mv1Lu cells are transfected with H-Ras^{V12}, the TGFβ-induced activation of A3-Luc is strongly inhibited as well (data not shown). This indicates a decreased level of TGFβ response in EpRas cells is not an accumulated Ras-independent defect in these cells but rather is in direct correlation with oncogenic Ras activity.

Finally, the Smad3(4AP) construct was used to investigate whether TGFβ responses lost in EpRas cells could be restored by a Ras-resistant Smad3 mutant. A3-Luc activation by TGFβ in EpH4 cells is only slightly enhanced by transfection of Smad3 or Smad3(4AP) vectors (Fig.46; Kretzschmar et al., 1999). However, transfection of Smad3(4AP) into EpRas cells strongly enhances the otherwise limited response of A3-Luc to TGFβ, whereas transfection of the wild-type Smad3 has only a small effect. From these results it can be concluded that oncogenic Ras: 1) increases phosphorylation of Smad2 and Smad3 in the linker region; 2) part or all of the phosphorylation of Smad2 and Smad3 at the 4S(T)P

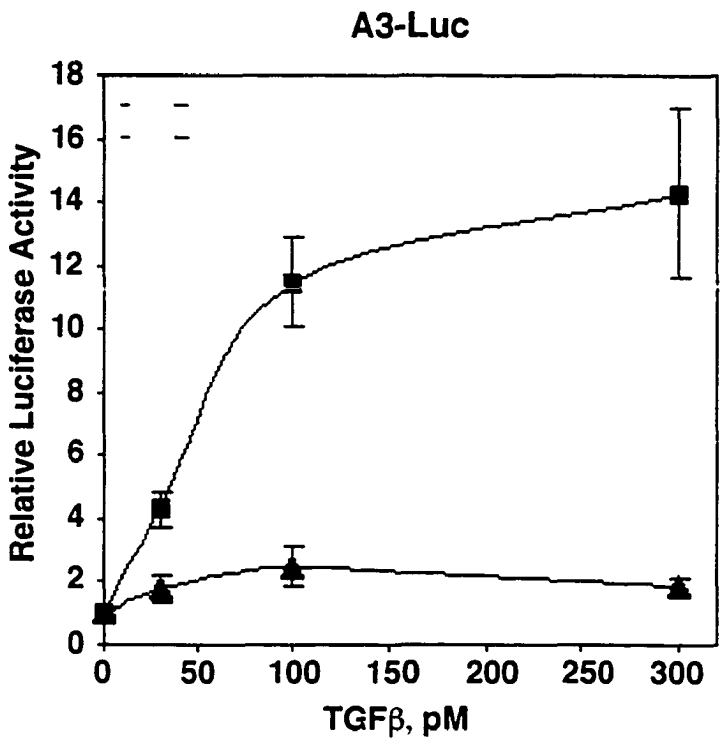

Figure 45. Oncogenic H-Ras inhibits Smad-dependent TGFβ transcriptional response. Activation of the A3-Luciferase reporter construct by the indicated concentrations of TGFβ was analyzed in EpH4 and EpRas mammary epithelial cells. (▲) EpH4; (■) EpRas.

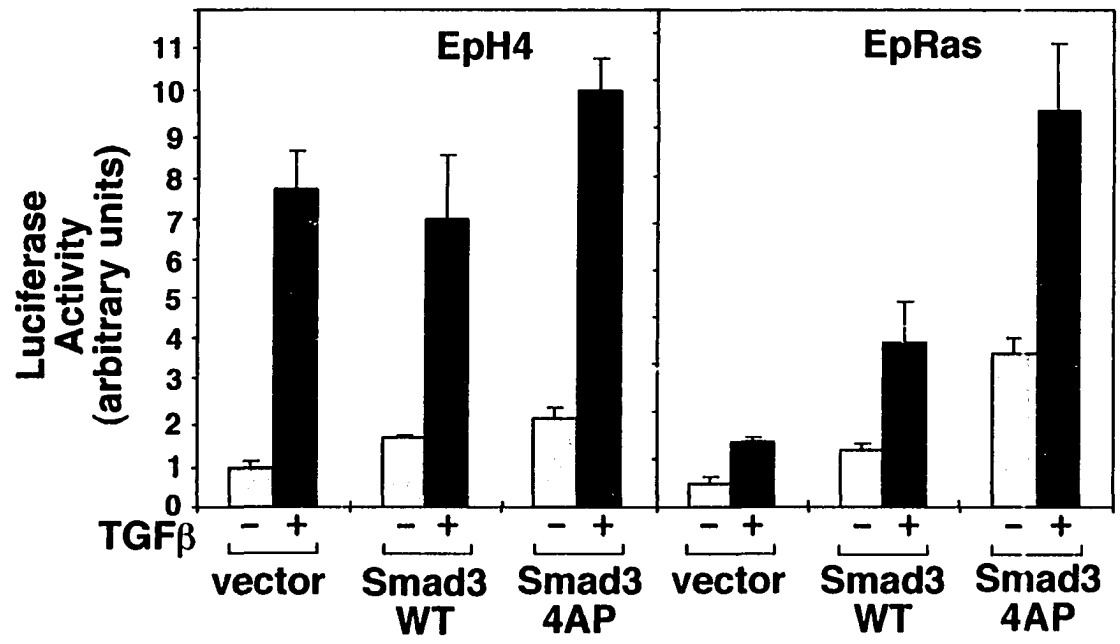

Figure 46. Ras-resistant Smad3 rescues TGFβ transcriptional responses in EpRas cells. EpH4 and EpRas cells were transfected with wild-type Smad3, Ras-resistant (4AP) Smad mutant, or the corresponding empty vector. These vectors were cotransfected with the Smad-responsive ARE-luciferase reporter construct (A3-Luc) and Fast2. Cells were incubated with or without TGFβ and luciferase activity was measured. Data are the average of triplicates ± S.D.

sites leads to inhibition of nuclear accumulation; and 3) ultimately inhibits transcriptional activation of TGFβ-responsive elements.

Phosphorylation of Smad by oncogenic Ras may play a role in tumor progression

TGFβ signaling is lost in some cancers by mutational inactivation of TGFβ signal-transduction components (for review, see Hata et al., 1998b; Kim et al., 2000). Other tumors retain the normal TGFβ receptors but have lost the capacity for growth arrest in response to TGFβ. Such a state of altered TGFβ responsiveness is observed in Ras-transformed cells. These cells typically exhibit a limited growth inhibitory response to TGFβ (Longstreet et al., 1988; Schwarz et al.,1988; Houck et al.,1989; Valverius et al., 1989; Filmus and Kerbel,1993). One possible mechanism for inhibiting TGFβ signaling in tumor cells, which have an oncogenic Ras, may be by inhibiting Smad2/3 nuclear accumulation.

To investigate this possibility several human colon cancer cell lines were treated with TGFβ to check for nuclear translocation of Smad2/3 (Table2,Fig.47). In those tumor cell lines which carry an oncogenic Ras, there is little to no nuclear translocation due to the presence of TGFβ. However, colon cancer cell lines which have only a wild-type Ras have a strong to partial response to TGFβ-induced nuclear translocation. The results suggest that an activated oncogenic Ras inhibits Smad2/3 nuclear accumulation in tumor lines which makes them refractory to TGFβ. When H-Ras^{V12} is transfected into the two TGFβ-sensitive

Cell line	*ras* allele[a]	Other alterations[a]	Smad2/Smad3, % nuclear		
			nontransfected	vector	H-Ras^{V12}
HT-29	wild type	*p53/APC⁻/src*	93	75	12
Colo205	wild type	*p53/APC⁻/src/lck/myb*	45	48	4
SW620	K-*ras*^{V12}	*p53/APC⁻/src/myb*	10	N.T.	
N.T.					
LoVo	K-*ras*^{D12}	*p53/APC⁻/src*	<2	N.T.	
N.T.					
DLD-1	K-*ras*^{D13}	*p53/APC⁻*	<2	N.T.	N.T.

[a]*ras* status and other known oncogenic alterations according to Sepp-Lorenzino et al. (1995). *p53* and *APC* are inactivated by mutations or not expressed. The oncogenes listed are mutated or overexpressed.

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Table 2. TGFβ-induced nuclear accumulation of Smad2/3 in human colon cancer cell lines. Cells were incubated with 10pM TGFβ for 30 min and subjected to anti-Smad2/3 immunofluorescence. The percentage of cells with predominant nuclear staining in the total nontransfected population or in the transfected (GFP positive) population is shown. (N.T.) Not tested.

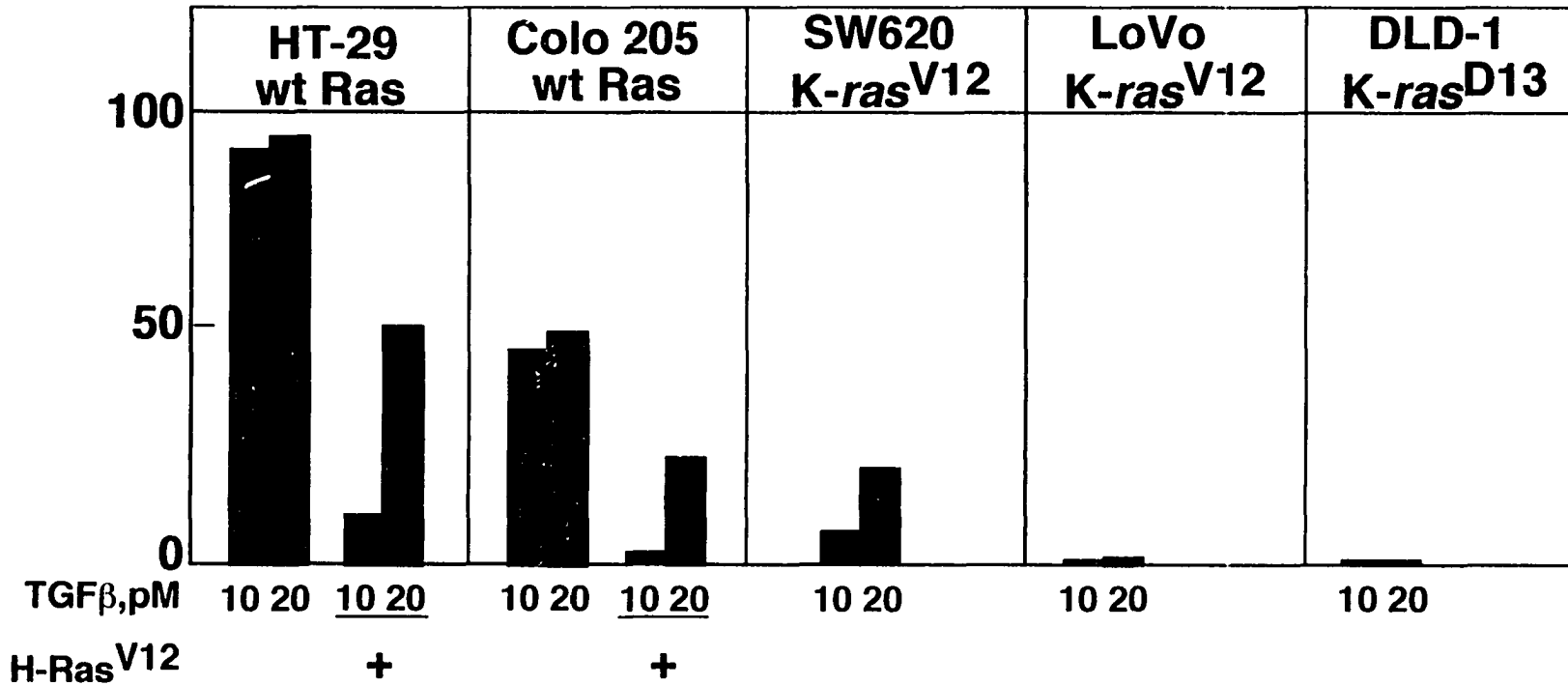

Figure 47. Nuclear accumulation of Smad2/3 in colon carcinoma cell lines. Cells were either not transfected (light grey bars) or transfected with a H-Ras^{V12} vector (dark grey bars) and treated with the indicated amount of TGFβ for 30 min. Cells were subjected to anti-Smad2/3 immunoflourescence and the percentage of cells with predominant nuclear staining are indicated. Headings specify the carcinoma cell line and it's *ras* status.

colon cancer cell lines, the cell lines also show a decreased nuclear accumulation of Smad2/3 in the presence of TGFβ. The inhibitory mechanism of oncogenic Ras on TGFβ signaling may allow the emergence of a tumorigenic phenotype and may explain why numerous tumors become refractory to TGFβ.

IFNγ uses an additional repressive mechanism

Dr. Luis Ulloa studied the effects of IFNγ on TGFβ signaling but was having difficulties in obtaining data showing effects of IFNγ on Smad phosphorylation. Because of my experience in this area I was assigned to the project by Dr. Joan Massague. The data obtained before my coming onto the project was fragmented, requiring me to utilize my insight and experience to redesign the experiments. Fortunately, I obtained results verifying phosphorylation of Smad1 by IFNγ and its effect on cellular localization which resulted in the data used in Figs. 50, 51, 52, 53 and 55. Dr. Luis Ulloa went on to show that this effect was rnediated by Smad7 which I present at the end of this section as a conclusion to this aspect of the project. These data were incorporated into:

> Ulloa, L., Doody,J. and J.Massague (1999). Inhibition of transforming growth factor-β/SMAD signalling by the interferon-γ/STAT pathway. Nature, 397: 710-713.

IFNγ inhibits Smad activation

Besides EGF and other RTK inducers, IFNγ is also known to be an antagonist of TGFβ in diverse cellular functions. TGFβ is a well-known immunosuppressor, although in some cases it can induce maturation of specific leukocytic cells (for review, see Letterio and Roberts, 1998). For example, TGFβ inhibits the production of IFNγ by T cells, suppressing T_{H1} development (Schmitt et al., 1994). A reciprocal effect occurs as well, where IFNγ suppresses TGFβ action, allowing for differentiation and activation of resting monocytes (McCartney-Frances and Wahl, 1994). TGFβ can also modulate expression of adhesion molecules necessary for leukocytic chemotaxis. TGFβ increases production of fibronectin and laminin receptors in monocytic cells whereas IFNγ inhibits the effects by TGFβ, decreasing fibronectin receptors and hence cellular adhesion (Bauvois et al., 1992). Therefore, in many circumstances, IFNγ and TGFβ are responsible for opposing actions in immune responses.

To investigate the interplay between the IFNγ/STAT and TGFβ signal transduction pathways, a genetically defined cell system previously used to dissect IFNγ signaling through Jak1 and Stat1 was utilized (Stark et al., 1998). The U4A human fibroblast cell line is derived from 2fTGH cells which have been chemically mutagenized to express a non-functional truncated Jak1 (Pelligrini et al., 1989; McKendry et al., 1991). Responsiveness to IFNγ can be restored by stable expression of exogenous Jak1, as in the stabily transfected subclone U4A/Jak1 (Muller et al., 1993). In both cell lines, when a TGFβ-inducible

reporter, A3-Luc, is transiently transfected in along with FAST2 there is an activation of the reporter by TGFβ (Fig.48; Ulloa et al., 1999). Both U4A and U4A/Jak1 have a functional TGFβ signaling pathway unaffected by the chemical mutagenesis of U4A cells. However, when IFNγ is added in addition to TGFβ there is a noticeable decrease in luciferase activity in U4A/Jak1 cells indicating that IFNγ inhibits TGFβ induction of the reporter A3-Luc. This inhibition is not seen in U4A cells, implying a requirement for a functional IFNγ signaling pathway to impede TGFβ signaling. IFNγ by itself has no effect on the A3-Luc reporter.

Could the inhibition by IFNγ of TGFβ signaling use the same mechanism as that utilized by EGF to inhibit TGFβ? To answer this question monoclonal antibodies that could distinguish between Smad2 and Smad3 were obtained (Fig.49). U4A cells express Smad3 but no detectable Smad2. To investigate whether Smad activation occurs upon TGFβ addition, cellular localization of Smad3 was visualized in U4A cells. Nuclear accumulation is inhibited by IFNγ in U4A/Jak1 cells but not in U4A cells (Fig.50). IFNγ alone does not have any discernible effect on the subcellular distribution of Smad3. Also, IFNγ is more effective against 20pM TGFβ than against 100pM (data not shown) indicating the extent of Smad3 activation may be determined by the balance of TGFβ and IFNγ signals. This effect is reminiscent of the results seen with EGF where higher additions of TGFβ can overcome inhibition by the mitogen. These results argue that IFNγ, signaling through Jak1 inhibits the TGFγ-induced nuclear accumulation of Smad3

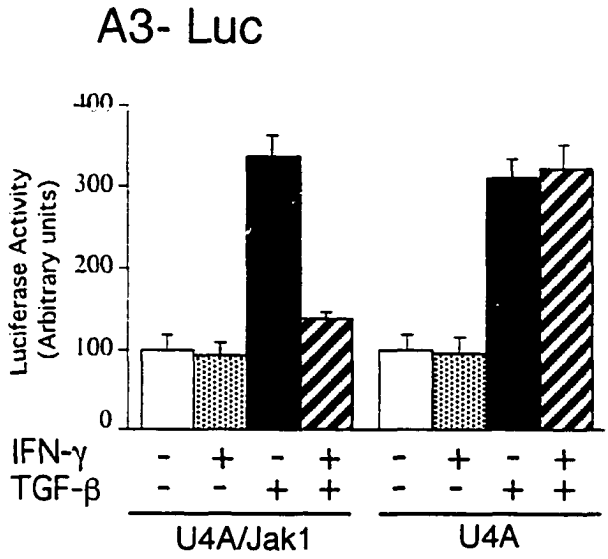

Figure 48. Inhibition of TGFβ transcriptional response by IFNγ. Response of the reporter gene A3-Luc to TGFβ in U4A and U4A/Jak1 cells. Inhibition of these responses by IFNγ. Values are averages of triplicate determination ±s.d.

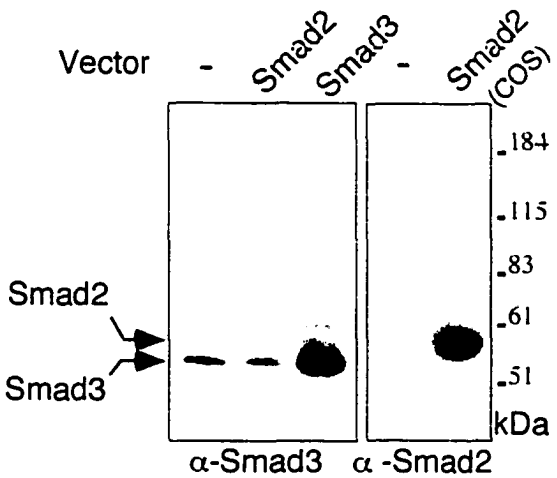

Figure 49. Levels of Smad2 and Smad3 as determined by using anti-Smad3 antibodies. U4A/Jak1 cells transfected with Smad vectors or empty vector were subjected to immunoblotting with Smad3-specific antibodies (left panel). Lysates from U4A/Jak1 cells (right panel, left lane) and Smad2-transfected COS cells (right panel, right lane) were subjected to anti-Smad immunoblotting.

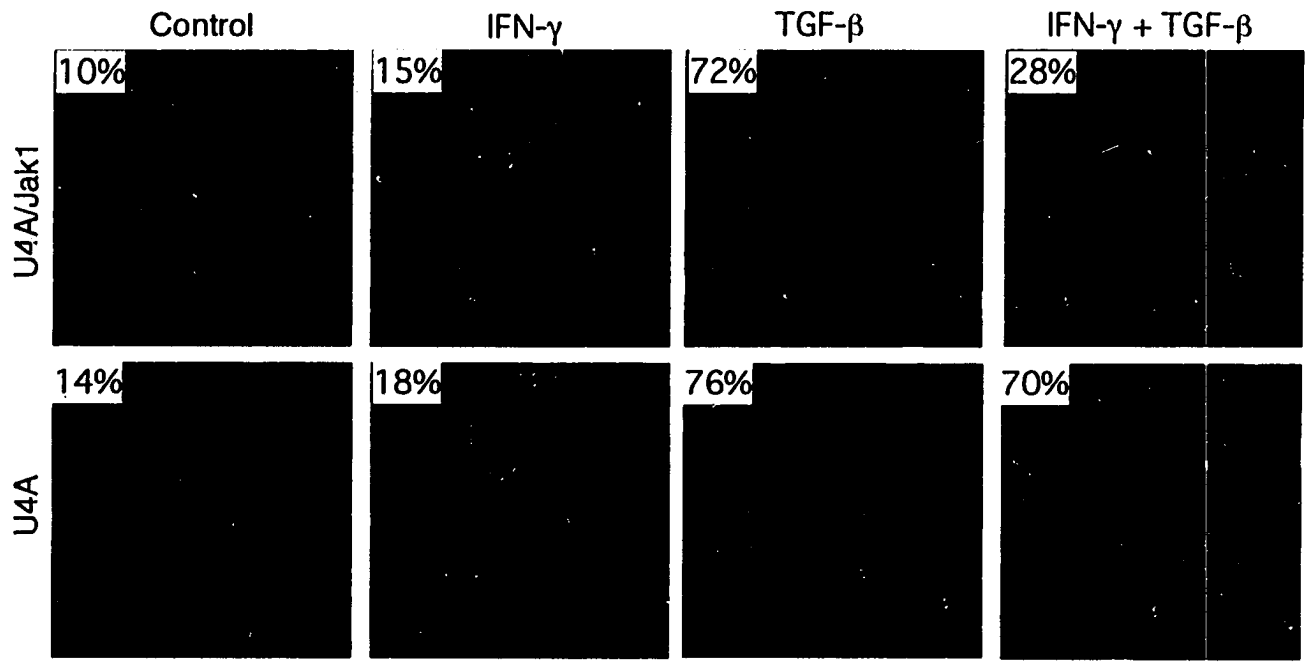

Figure 50. Smad3 immunofluorescence in cells incubated with IFNγ and/or TGFβ. U4A and U4A/Jak1 cells were incubated with IFNγ for 15 min where indicated prior to TGFβ addition. 10pM TGFβ was added 30 min before innumostaining with anti-Smad3 antibodies and FITC-conjugated secondary antibodies. The same slides were counterstained with DAPI to visualize nuclei. The percentage of cells showing predominantly nuclear staining are indicated.

Smad phosphorylation increases with IFNγ addition

Phosphorylation of Smad by Erk2 may be the mechanism by which IFNγ also inhibits TGFβ signaling, similar to that seen with EGF. IFNγ increases activity of Erk in U4A/Jak1 cells (Sakatsume et al., 1998). ^{32}P-labeled U4A/Jak1 cell lysate immunoblotted against Flag-Smad3 show an increase in phosphorylated Smad3 within 15 minutes of IFNγ treatment (Fig.51). HCl digestion of the ^{32}P-labeled Smad3 subjected to phosphoamino acid analysis reveal the majority of the phosphorylations occurs on serine residues (Fig.52) and therefore is not a direct phosphorylation by Jak1 tyrosine kinase. The phosphorylation of Smad3 in the presence of IFNγ is suggestive of the phosphorylation of Smad3 by EGF where the mitogen is responsible for the phosphorylation of Smads in the linker region at specific S(T)P sites. To investigate whether the same mechanism is at work with IFNγ, the wild-type Smad 3 or the Smad3(4AP) mutant was transfected into U4A/Jak1 cells and cells were subjected to IFNγ (Fig.53). As shown previously, IFNγ induces an increase in Smad3 phosphorylation within 15 minutes of addition of the mitogen. The mutation of the 4S(T)P motifs abolishes IFNγ-induced Smad3 phosphorylation as well as basal phosphorylation due to low level serum induction. When MEK inhibitor is added to U4A/Jak1 cells before IFNγ treatment, there is a loss of Smad3 phosphorylation (data not shown) demonstrating that Erk is responsible for this phosphorylation of Smad3 in response to IFNγ. These results indicate that IFNγ induces Smad3 phosphorylation by Erk at the 4S(T)P sites.

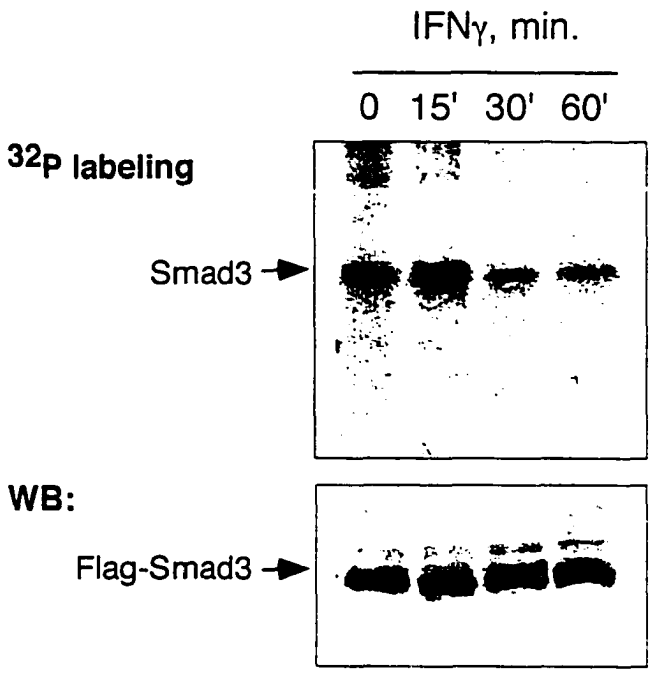

Figure 51. IFNγ induces phosphorylation of transfected Flag-Smad3 in U4A/Jak1. U4A/Jak1 cells were transfected with Flag-tagged Smad3 and labeled with ^{32}P. Cells were incubated with IFNγ for the times indicated before lysis. Flag-Smad3 was immunoprecipitated with anti-Flag antibodies. Smad3 expression was monitored by anti-Flag immunoblotting.

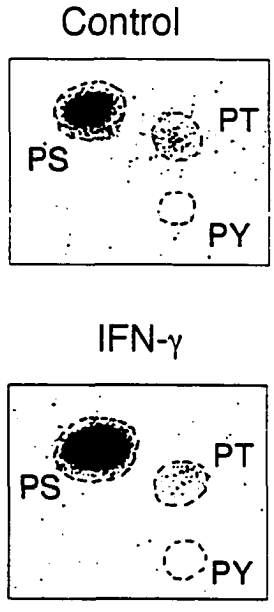

Figure 52. Smad3 is phosphorylated mainly on serines. ^{32}P-labeled Flag-Smad3 from cells treated or not treated with IFNγ for15 min was subjected to phosphoamino acid analysis. Positions shown correspond to phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY).

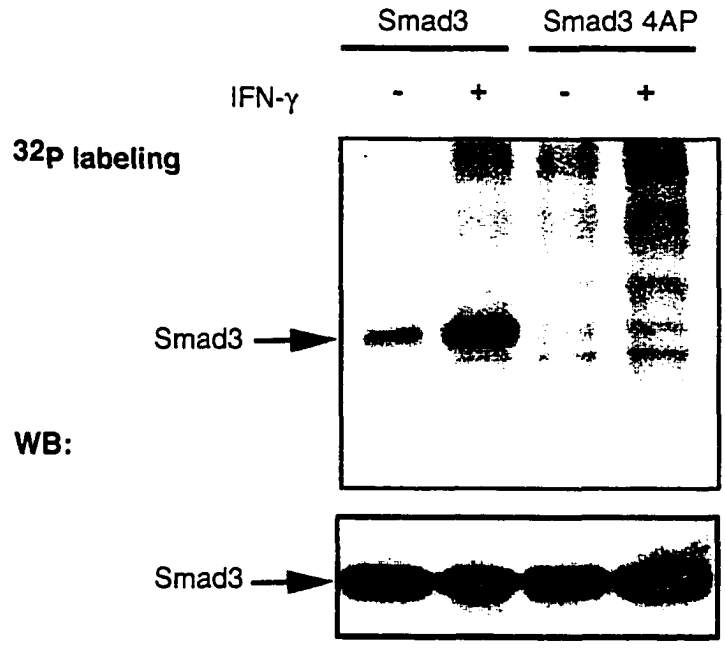

Figure 53. Phosphorylation of Smad3 by IFNγ is in the linker region. U4A/Jak1 cells were transiently transfected with either wild-type Flag-Smad3 or the mutant Flag-Smad3 (4S(T)P to 4A(V)P). ^{32}P-labeled cells were incubated for 15 min with IFNγ as indicated and immunoprecipitated with anti-Flag antibodies. Smad3 levels were monitored by anti-Smad3 western immunoblotting.

Even though MEK inhibitor prevents IFN-induced Smad3 phosphorylation, MEK1 inhibitor does not prevent the IFNγ-mediated decrease of the A3-Luc response (Fig.54, Ulloa et al., 1999). TGFβ addition stimulates luciferase activity in U4A/Jak1 cells from a TGFβ responsive promoter, A3-Luc, but luciferase activity is inhibited when TGFβ is coincubated with IFNγ. When U4A/JAK1 cells are incubated with MEK1 inhibitor prior to IFNγ treatment, the MEK1 inhibitor does not rescue cells from IFNγ inhibition. These results indicate that IFNγ may induce Smad3 phosphorylation by Erk but that this phosphorylation is not the predominant requirement for IFNγ-mediated inhibition of TGFβ signaling. However, in both cases where MEK1 inhibitor is added to cells stimulated with either TGFβ alone or in combination with IFNγ, TGFβ-induced luciferase activity increases. This indicates that although phosphorylation of Smad3 by Erk1 is not the main component of IFNγ inhibition it plays a role in decreasing TGFβ-mediated transcriptional activity.

IFNγ affects TGFβ-induced phosphorylation

Observations indicate that the IFNγ-induced inhibition of TGFβ signaling may be due to Smad3 phosphorylation, not by Erk1 but by the TGFβ receptors. Short preincubation with IFNγ inhibits TGFβ-induced phosphorylation of both endogenous (Fig.55) and transfected Smad3 (Fig.56) in U4A/Jak1 cells. Smad3 phosphorylation by TGFβ receptor I in the presence of IFNγ is equal to that seen with IFNγ alone, indicating that there is no TGFβ-induced phosphorylation of

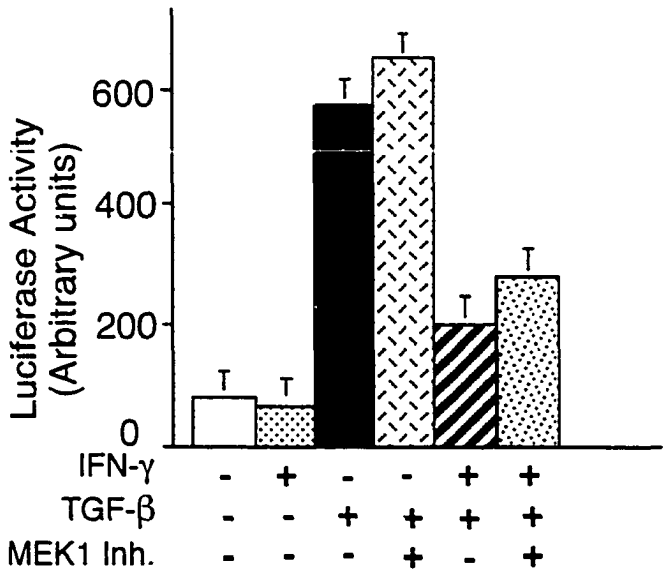

Figure 54. Erk phosphorylation is not required for Smad inhibition by IFNγ in U4A/Jak1 cells. Cells were transfected with the A3-Luc luciferase reporter construct. Activation of cells with the indicated agents was done 16 hours before lysis and luciferase activity measurements. Values are averages of triplicate determination ± s.d.

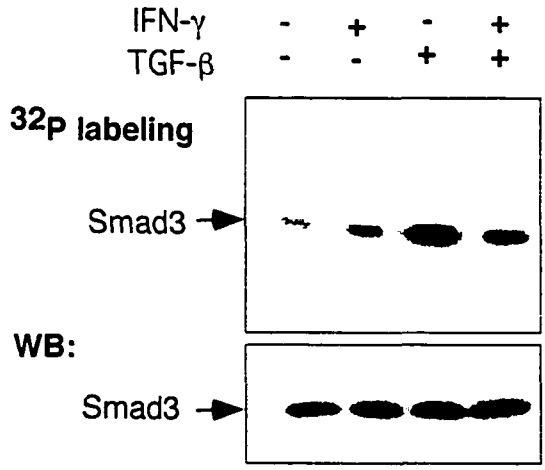

Figure 55. Phosphorylation of endogenous Smad3 in ^{32}P-labeled U4A/Jak1. Cells were incubated for 1 hour with IFNγ or TGFβ as indicated before subjecting to ^{32}P-labeling. Smad3 was immunoprecipitated with anti-Smad3 antibodies for either phosphate labeling or Western immunoblotting.

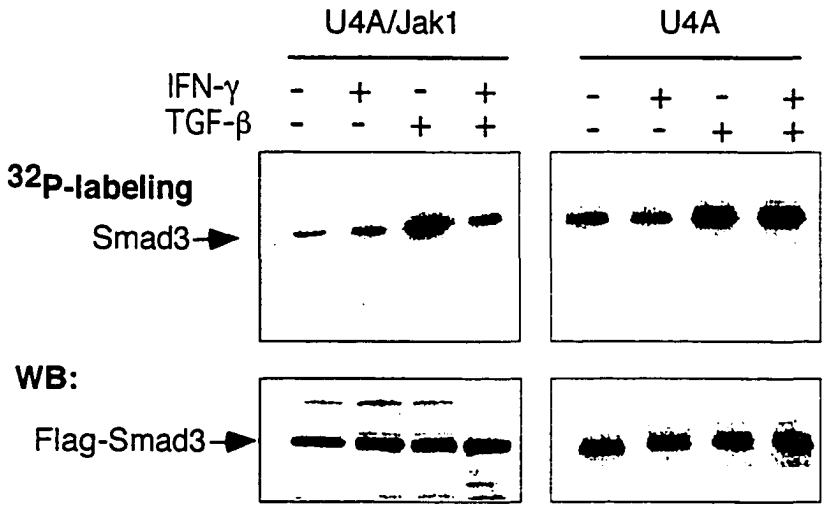

Figure 56. Phosphorylation of Flag-Smad3 is inhibited by IFNγ. U4A and U4A/Jak1 cells were incubated for 1 hour with IFNγ or TGFβ as indicated. Smad3 was immunoprecipitated with anti-Flag antibodies for either phosphate labeling or Western immunoblotting.

Smad3 under these conditions. This effect of IFNγ requires Jak1 since it was not observed in U4A cells which lack Jak1 (Fig.56). Inhibition of TGFβ-induced Smad3 phosphorylation by IFNγ is independent of Erk since it persists in the presence of MEK1 inhibitor (Fig.57). Therefore inhibition of TGFβ-mediated phosphorylation of Smad3 occurs via a different mechanism from Erk activation.

In light of these results, IFNγ effects on the TGFβ receptor I-Smad3 interaction were investigated. Inhibition of TGFBRI binding to Smad3 should prevent phosphorylation of Smad3 in response to TGFβ. Cell surface receptors were labeled by crosslinking to bound ^{125}I-labeled TGFβ and immunoprecipitated with anti-Flag antibodies against transfected Flag-Smad3 (Fig.58,Ulloa et al., 1999). Cells transfected with Flag-Smad3 resulted in a coprecipitation of Smad3 with TGFβ type I and type II receptors. With the addition of IFNγ there is a decrease of Smad3 bound receptor complex. This effect of IFNγ was not observed in U4A cells lacking Jak1. However, the levels of both TGFβ type I and type II receptors were not affected by the incubation of cells with IFNγ. Thus, IFNγ interferes with Smad3 association with its corresponding receptors.

Smad7 upregulation by IFNγ inhibits Smad3

IFNγ specifically inhibits an early step in the TGFβ-induced activation of Smad3. One possible mediator of such an effect is Smad7, a member of the Smad family, which interferes with TGFβ signaling. Smad7 binds to the TGFβ-receptor complex, preventing its interaction with and phosphorylation of Smads

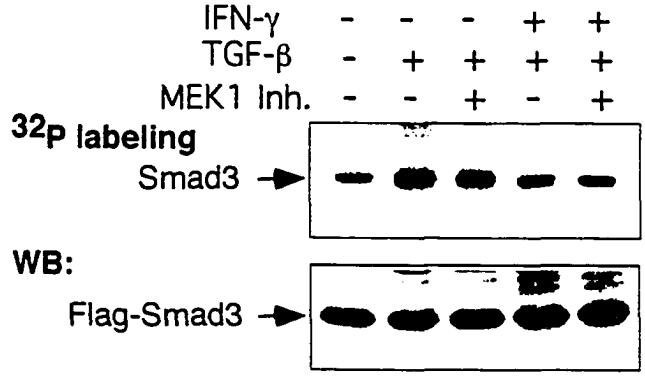

Figure 57. Phosphorylation of Flag-Smad3 in U4A/Jak1 cells incubated with cytokines. [^{32}P]-labeled cells were incubated with growth factors or inhibitors for the time indicated. Flag-Smad3 was immunoprecipitated with anti-Flag monoclonal antibodies for either phosphate labeling or Western immunoblotting.

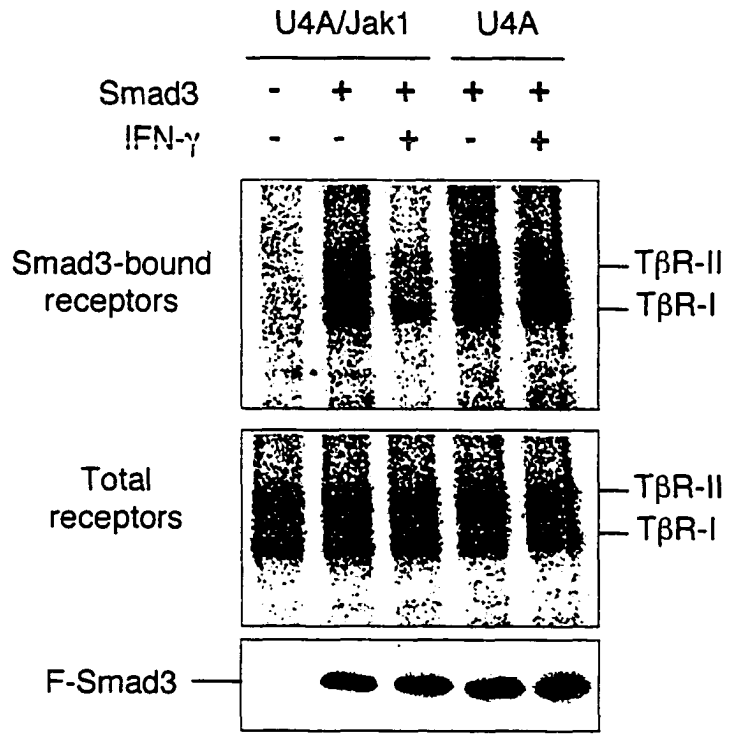

Figure 58. Receptor interaction with Flag-Smad3. Cells were transfected with Flag-Smad3 and incubated with IFN-γ. Cells surface receptors were labeled by crosslinking to bound ^{125}I-labeled TGFβ. Receptor interaction with Flag-Smad3 was determined by immunoprecipitation with anti-Flag antibodies (top). Aliquots of cell lysates were subjected to SDS-PAGE and autoradiography (middle panel). Other aliquots were subjected to anti-Flag western immunoblotting (bottom panel).

(Hayashi et al.,1997; Nakao et al.,1997b). Indeed, IFNγ induced expression of Smad7 in U4A/Jak1 cells increases the messenger RNA levels at least ten-fold over the basal level, which was barely detectable (Fig.59, Ulloa et al., 1999). This increase was maximal 30 minutes after IFNγ addition and did not occur in U4A cells lacking Jak1. IFNγ does not affect the expression of Smad6 or FKBP12, both of which inhibit TGFβ receptor activation (data not shown). These results indicate that *Smad7* induction by IFNγ is a specific and direct gene response mediated by the Jak1 pathway.

Smad7 inhibits the association of TGFβ receptors with Smad3 by binding to the TGFβ type I receptor, limiting access of Smad3 to the receptor (Hayashi et al.,1997; Nakao et al,1997b; Souchelnytskyi et al., 1998). To seek evidence of this effect in cells treated with IFNγ, ^{125}I-labeled receptors were checked for association with Smad7. IFNγ increases Smad7 protein levels as determined by Western immunoblotting with anti-Smad7 antibodies (Fig.60, Ulloa et al., 1999). Correlating with this increase, IFNγ raises the level of Smad7 protein associated with the receptor complex to prevent Smad3 phosphorylation by the TGFβ type I receptor.

That inhibition of TGFβ-induced phosphorylation of Smad3 is directly correlated to IFNγ induction of Smad7 can be evaluated by the use of antisense oligonucleotides. Cells treated with *Smad7* antisense oligonucleotides prevent IFNγ inhibition of Smad3 phosphorylation by the TGFβ type I receptor (Fig.61, Ulloa et al., 1999). The corresponding sense oligonucleotide and a different

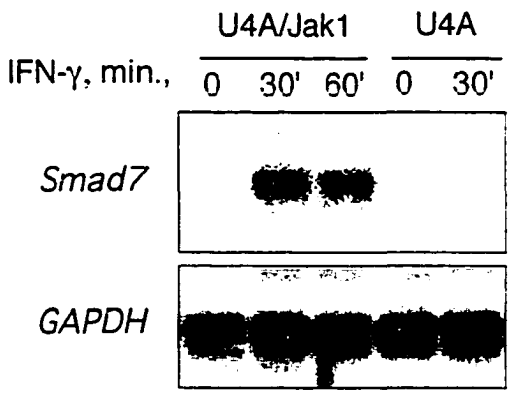

Figure 59. Induction of an antagonistic Smad by IFNγ . U4A/Jak1 cells were incubated with IFNγ for the indicated times and *Smad7* mRNA levels were determined by Northern blotting of poly(A+) RNA. To control for mRNA loading, the same blots were probed for *glyceraldehyde-3-phosphate dehydrogenase (GAPDH).*

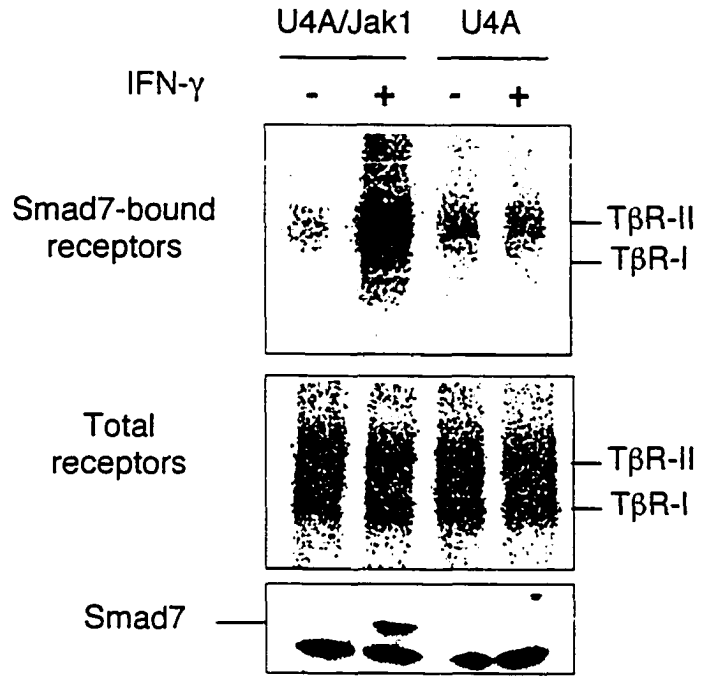

Figure 60. Interaction between TGFβ receptors and Flag-Smad7.

Cells were incubated with IFN-γ. Cells surface receptors were labeled by crosslinking to bound ^{125}I-labeled TGFβ. Receptor interaction with Smad7 was determined by immunoprecipitation with anti-Smad7 antibodies (top). Aliquots of cell lysates were subjected to SDS-PAGE and autoradiography (middle panel). Other aliquots were subjected to anti-Flag western immunoblotting (bottom panel).

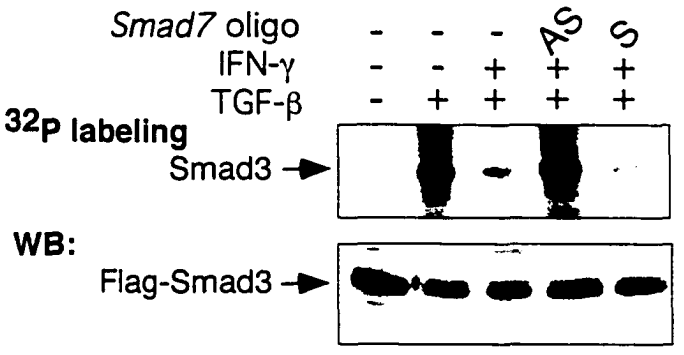

Figure 61. Effect of *Smad7* antisense oligonucleotide on Smad3 phosphorylation. Cells were cotransfected with Flag-Smad3 vector and the indicated *Smad7* oligonucleotides, and then incubated with or without IFNγ for 45 min before 45 min with or without TGFβ.

antisense oligonucleotide, both of which did not prevent the increase in Smad7, failed to prevent the inhibition of Smad3 phosphorylation by IFNγ (Fig.61, data not shown). Therefore, IFNγ signaling through Jak1 rapidly increases the expression of an anti-Smad, Smad7, causing the inhibition of TGFβ-mediated Smad3 phosphorylation, loss of TGFβ signaling to the nucleus and an attendant loss of activation of TGFβ-inducible genes.

Discussion

The identification of Mad in *Drosophila*, sma in *C.elegans*, and Smad1 in vertebrates provided the first major downstream component after the BMP receptors involved in BMP signaling. The mechanism by which Smad1 is activated and how it transmits the BMP signal needed to be elucidated since the genetic screens used to identify Mad and sma in *Drosophila* and *C.elegans* gave no clue as to their mechanism of action. It is known that Smad1 is phosphorylated in response to BMP2 and BMP4 (Hoodless et al., 1996; Yingling et al., 1996), however, the identity of the kinase and the functional relevance of this phosphorylation remained unknown until our mutational analysis of Smad1 (Kretzschmar et al., 1997a).

Smad1 phosphorylation by BMPRI leads to BMP-induced transcriptional activation

Smad1 is a direct substrate for the BMP type1 receptor.

Smad1 is rapidly phosphorylated in response to BMP, supporting earlier evidence of Smad1 activation by phosphorylation (Hoodless, et al., 1996). This phosphorylation is mainly on serines and occurs within 30 minutes of BMP addition, suggesting that phosphorylation of Smad1 is an early event of BMP signaling. This led to the investigation of BMP receptors as a possible kinase for Smad1 phosphorylation. *In vitro* kinase assays involving immunoprecipitated activated BMPRIB (Weiser et al., 1995) and purified bacterially expressed

Smad1 led to a direct phosphorylation of Smad1 by BMPRIB at nanomolar concentrations. These results could not be repeated with activin receptor type I, suggesting phosphorylation is specific (Kretzschmar et al., 1997). Using highly purified preparation of bacterially expressed BMPRIB, Smad1 is also phosphorylated *in vitro*. This provides strong evidence that BMPRIB has intrinsic Smad1 kinase activity. It also suggests that BMPRIB rather than a coimmunoprecipitated non-relevant kinase from COS-1 cells phosphorylates Smad1.

Smad1 is phosphorylated on the carboxy-terminal SSXS in response to BMP

Several lines of reasoning led to the investigation of the carboxy-terminal tail of Smad1 as the possible site of BMPRIB kinase activity. First, tryptic digestion of ^{32}P-labeled Smad1 shows only one band of BMP-induced phosphorylation. This phosphorylation is not in the linker region that shows up as a 13kD fragment, but in a smaller fragment which can either be part of the N- or C- terminal region. Secondly, certain mutations in the carboxy-terminal domains of Smad1 and Smad2 can inhibit BMP and TGFβ-induced phosphorylation (Eppert et al., 1996; Hoodless et al., 1996), indicating that the location of the phosphorylated serines may be in this region. And lastly, when a Smad1 carboxy-terminal domain is transfected into R1B/L17 cells, the construct becomes phosphorylated in response to BMP (Kretzschmar et al., 1997a).

Mutational analysis of the carboxyl-terminus identified the SSVS motif at the terminus of Smad1 as the BMP2-dependent phosphorylation site. Substituting alanines for serine residues completely abolishes the BMP-induced phosphorylation of Smad1. Interestingly, substitution of any of these serines with alanine completely eliminates BMP-induced phosphorylation indicating that any modification of Smad1 structure hinders the phosphorylation of the other two serines. Crystal structure of the carboxyl-terminus of Smad4, although not resolving the last few amino acids, indicates that this terminal tail lies outside the conserved core structure of the MH2 domain (Shi et al., 1997). Phosphorylation of this exposed tail is unlikely to affect the overall structure of the protein. Analysis of Smad2 phosphorylation by TGFβ has shown a requirement for the SSXS motif for Smad2 phosphorylation (Marcias-Silva et al., 1996). This motif is also present in Smad3, Smad5 and Smad8, all of which are signaling Smads. This motif is conspicuously absent from Smad4, which does not become phosphorylated in cells treated with TGFβ or BMP (Lagna et al, 1996; Zhang et al., 1996; Yingling et al., 1997) and Smad6 and Smad7, which inhibit Smad signaling. Therefore, the SSXS motif at the carboxy-terminal domain of signaling Smads is a common motif for phosphorylation of these Smads by their respective receptors. Once Smad is activated by its receptor it associates with Smad4 (Lagna et al., 1996; Zhang et al., 1996), which is constitutively phosphorylated by an unknown mechanism, in a receptor-independent manner. Smad4 association

with the receptor-regulated Smad1 is dependent on Smad1 becoming phosphorylated by BMPRI at the SSVS motif (Kretzschmar et al., 1997a)

Mutation of the SSVS motif to AAVA prevents BMP-induced nuclear translocation of Smad1 and transcriptional activation. This study used the carboxy-terminal domain of Smad1 fused to the DNA-binding domain of GAL4 since no BMP responsive genes were characterized. Subsequently, the Xvent2 promoter from *Xenopus* has been sequenced and Smad1 has been shown to bind directly to the promoter in conjunction with a Smad co-activator OAZ (Onichtchouk et al, 1998; Hata et al., 2000). Smad1 therefore moves into the nucleus once phosphorylated, and binds to DNA to activate transcription.

Present evidence argues that serine-threonine kinase receptors signal to the nucleus by a relatively simple mechanism. There is only one protein necessary to transmit signals from the receptor to the genes which they control thereby shortening our research efforts in the field by many years. This is in contrast to tyrosine kinase receptors that utilize multiple steps and have kept their researchers happy for decades.

In short, BMP binds to a complex of two receptor kinases, BMPRII and BMPRI. BMPRII phosphorylates BMPRI, which in turn phosphorylates Smad1. Phosphorylated Smad1 associates with Smad4 and subsequently, in the nucleus, interacts with a sequence specific DNA-binding protein such as OAZ (Hata et al., 2000) to activate a variety of biological responses.

EGF-dependent Smad phosphorylation leads to inhibition of TGFβ and BMP signaling

EGF induces Smad1 linker domain phosphorylation at SP sites

BMP-dependent phosphorylation of Smad1 occurs at the carboxy-terminal tail but there is also a basal phosphorylation of Smad1 in the linker region that is BMP-independent. Mutation of various serines to alanines in this region has no effect on phosphorylation of Smad1 except for the mutant Smad1(4AP) which mutates the four repeated PXSP motifs. Smad2 and Smad3 have 4 S(T)P sites as well, but not all sites have an adjoining proline two residues N-terminal. Mutation of the S(T)P sites in Smad2 and Smad3 results in the same phenomenon as Smad1, namely decrease in basal phosphorylation. We were unable to distinguish whether these sites are phosphorylated individually since the reduction in phosphorylation is slight with only one mutation of the SP sites. However, mutations of fewer sites caused a proportional reduction of phosphorylation, which would suggest that they are all phosphorylated.

Since PXSP motifs are consensus sites for MAP kinases (Clark-Lewis et al., 1991; Gonzalez et al., 1991) various growth factors, mitogenic proteins and inhibitors were tested to pinpoint which factors influence Smad phosphorylation of the linker regions. In summary, mitogens that utilize MEK1, Erk2 and Ras such as EGF and hepatocyte growth factor (HGF)(Kretzschmar et al., 1997b) phosphorylated Smad1, Smad2 and Smad3. EGF causes a rapid rise in phosphorylation of Smad that is transient, reminiscent of a direct signaling event.

This EGF and Ras induction of Smad phosphorylation was abolished by mutation of the four SP sites pinpointing the sites of phosphorylation to the SP sites of the linker region. Mitogens, which stimulate the stress-activated MAP kinases JNK and p38, have no effect on phosphorylation of the Smad linker region. Nor does the use of wortmannin, an inhibitor of the PI(3) kinase pathway, have significant effects on Smad phosphorylation. Taken together, these results provide strong evidence that Smad1, Smad2 and Smad3 are targets of the Erk subfamily of MAP kinases in the cell. Consistent with this, basal phosphorylation of Smad is reduced upon serum starvation, a condition that decreases Erk activity. Evidence is also provided to show Erk kinases directly phosphorylate Smads at the SP sites by using purified Smads and the mutant Smad(4SP) as substrates in *in vitro* assays containing activated Erk2. Titration of wild-type Smad1 with activated Erk2 demonstrates that Smad1 is a substrate for Erk2 at nanomolar concentrations. Results from the mutant Smad1(4SP) demonstrate that phosphorylation of Smad1 *in vitro* occurs at the same sites that are phosphorylated in response to EGF *in vivo*.

EGF and HGF have been shown to phosphorylate Smad2 at the C-terminal SSMS in HepG2 cells (deCaestecker et al., 1998). Increase in EGF- and HGF-mediated Smad2 phosphorylation in Mv1Lu and R1B/L17 cells in the paper concur with these results, showing a rapid but transient increase in phosphorylation. However, upon mapping the site of phosphorylation of Smad2 by phosphotryptic peptide maps, it was concluded that the spot corresponded to

a phosphorylation of the C-terminal tail (deCaestecker et al., 1998). Contradiction in the data may be attributed to a change of cell type, which may have differing responses to EGF or to the notoriously problematic interpretation of peptide maps that vary from plate to plate. Recently, mutation of Erk sites in the linker region of Smad1 has been shown to decrease Ras-induced phosphorylation of Smad1 in rat intestinal epithelial cells (Mulder, 2000), which supports these claims that EGF induces phosphorylation of the linker region and not the C-terminal tail of Smads. Furthermore, a constitutively active MEKK1 increased the phosphorylation state of Smad2 but not a Smad2 AAXA mutant in bovine endothelial cells, reinforcing the idea that phosphorylation is not at the C-terminus. MEKK1 has been shown to associate directly with Ras (Derijard et al., 1994; Russell et al., 1995) further bolstering the conclusion that the linker region is the target of mitogen phosphorylation.

Phosphorylation of the Smad linker region regulates cellular localization and transcriptional function.

The consequences of Smad phosphorylation by EGF are an inhibition of nuclear accumulation and subsequently, transcriptional activation. The following lines of evidence support the conclusion that Ras-induced phosphorylation of the 4SP sites prevents accumulation of Smads in the nucleus and inhibits signaling. First, EGF prevents BMP-induced transfected Smad1 and TGFβ-induced endogenous Smad2/3 from accumulating in the nucleus. Second, Smad nuclear

accumulation is inhibited by transfection of a constitutively active MEK1 and stimulated by the addition of a MEK1 inhibitor. Third, cells, which express a stably transfected H-Ras^{v12} have lower levels of Smad2/3 nuclear translocation in response to TGFβ. Finally, these EGF-, MEK1- and Ras-induced effects are prevented by mutation of the 4S(T)P sites in the linker region of Smads. This decrease in nuclear accumulation of Smads is not due to interference with Smad4 complex formation with the receptor-mediated Smad since this association is not disrupted by EGF (Kretzschmar et al.,1997b). Moreover, by using Smad4-defective cells, it was demonstrated that Smad1 and Smad2 association with Smad4 was independent of Smad1 and Smad2 nuclear translocation (Liu et al., 1997).

The decrease in nuclear localization of Smads correlates with a reduction in transcriptional activation. Gal4-Smad1 induction decreases when cells are incubated with both BMP and EGF compared to BMP alone, while A3-luc induction by TGFβ decreases in Ras-transfected cells. These transcriptional activities can be rescued when the corresponding cells are transfected with Smads containing a 4AP mutation in the linker region. However, rescue is weak since TGFβ activation of Smad3(4AP) is competing with endogenous Smad2 and Smad3.

These results conflict with those showing an increase in nuclear accumulation and transcriptional activation of Smads in response to EGF and other signaling pathways (deCaestecker et al., 1998; Engel et al., 1999). Both

papers show activation of a TGFβ reporter plasmid, p3TP-lux, in response to EGF. The 3TP-lux reporter has a TGFβ response element but also contains three AP-1 sites (Carcamo et al,1995). EGF can activate AP-1 directly through the Ras/MAPK pathway or by activators such as phorbol esters (Hunter and Karin, 1992; Davis, 1993). In many cases, increase in luciferase activity by TGFβ is concurrent with an increase in basal activity such that the fold induction over background (which would be a more accurate reflection of TGFβ activity) remains constant. Therefore increased TGFβ induction of the 3TP-lux reporter construct is actually an increase in basal phosphorylation. Furthermore, Ras signaling has a general effect on transcription (Abdellatif et al., 1994) and can stimulate general transcriptional coactivators (Xu et al., 1998). Three-fold increases in 3TP-lux luciferase activity have been obtained by EGF in the absence of TGFβ (data not shown).

While MEKK1 has been shown to increase both Smad2 nuclear accumulation and transcriptional activation in response to TGFβ, MEK1 in the same paper inhibits nuclear accumulation and TGFβ-induced responsive genes (Brown et al, 1999), substantiating our results with MEK1. Their activation by MEKK1 was not supported by incubation of cells with TNFα, a distinct activator of MEKK1. In bone endothelial cells TNFα failed to show any significant stimulation of Smad2-mediated transcription (Brown et al., 1999) and reports show that TNFα in fact inhibits TGFβ signaling (Snoeck et al., 1996; Verrecchia et al., 2000). We were unable to get any phosphorylation of Smad with TNFα. That the

4SP phosphorylation by MEKK1 may be a function of JNK activation is a possibility. JNK activation occurs as a secondary event in TGFβ induction, peaking 16-24 hours after TGFβ addition (Atfi et al., 1997a; Wang et al., 1997; Engel et al., 1999; Hu et al., 1999; Zhou et al., 1999). Whether JNK is involved in an early response to TGFβ is currently unclear and has generated numerous conflicting reports. These results do not support the notion that EGF or signaling molecules in mitogenic pathways other than members of the TGFβ family signals through Smads.

Activated Erk kinases are distributed throughout the cytoplasm and the nucleus allowing phosphorylation of Smads by Erk to occur in either compartment. There are two different mechanisms by which inhibitors of nuclear accumulation of Smads can be achieved. Erk may phosphorylate Smads in the cytoplasm, preventing Smads from translocating to the nucleus and therefore inhibiting induction of gene responses. Or, Erk may phosphorylate Smads in the nucleus, stimulating either nuclear export or nuclear degradation of the Smads and therefore, decrease the amount of Smads available for transcribing TGFβ-activated genes. The present results do not reveal the mechanism Erk uses to inhibit nuclear accumulation. Nuclear degradation of TGFβ-activated Smad2 by ubiquitin has been reported as a mechanism of quenching TGFβ signaling (Lo and Massague, 1999). Although lower nuclear levels of Smads in response to EGF are seen, there is a concomitant increase in cytoplasmic accumulation indicating there is no increase of Smad degradation. This would favor the

hypothesis that EGF inhibits nuclear translocation of Smads rather than increasing nuclear export.

Therefore, a model by which EGF interferes with TGFβ-signaling is proposed (Fig 62). It is shown that in the presence of EGF, TGFβ normally activates TGFβ receptors to phosphorylate Smad at the carboxyl-terminus and allow the receptor-activated Smads to associate with Smad4. However, by phosphorylating receptor-regulated Smads on the S(T)P sites located within the linker region, EGF either prevents Smads from being imported to the nucleus or accelerates the export of Smads from the nucleus, thereby inhibiting activation of TGFβ-inducible genes. Differential regulation of Smad1 by TGFβ and EGF provides a mechanism by which opposing effects of these factors affect cellular differentiation and vertebrate development. During development of tracheal placodes in *Drosophila,* EGF forms the dorsal trunk and visceral branch. Dpp (the *Drosophila* homologue of Smad1), in contrast, induces the tracheal pit. Mutations in either the EGF or Dpp pathways demonstrate antagonistic interactions between the two (Wappner et al., 1997). A phenomenon that Wappner observed was when the strength of the Dpp pathway was reduced, EGF was able to recruit cells from the dpp domain into an EGF-dependent fate. However, at normal dpp levels, EGF was unable to influence tracheal development. In this report a similar result was noted, where EGF inhibited TGFβ-inducible Smad nuclear accumulation when TGFβ levels were low but as TGFβ dosages were increased, a proportional decrease in EGF effect was

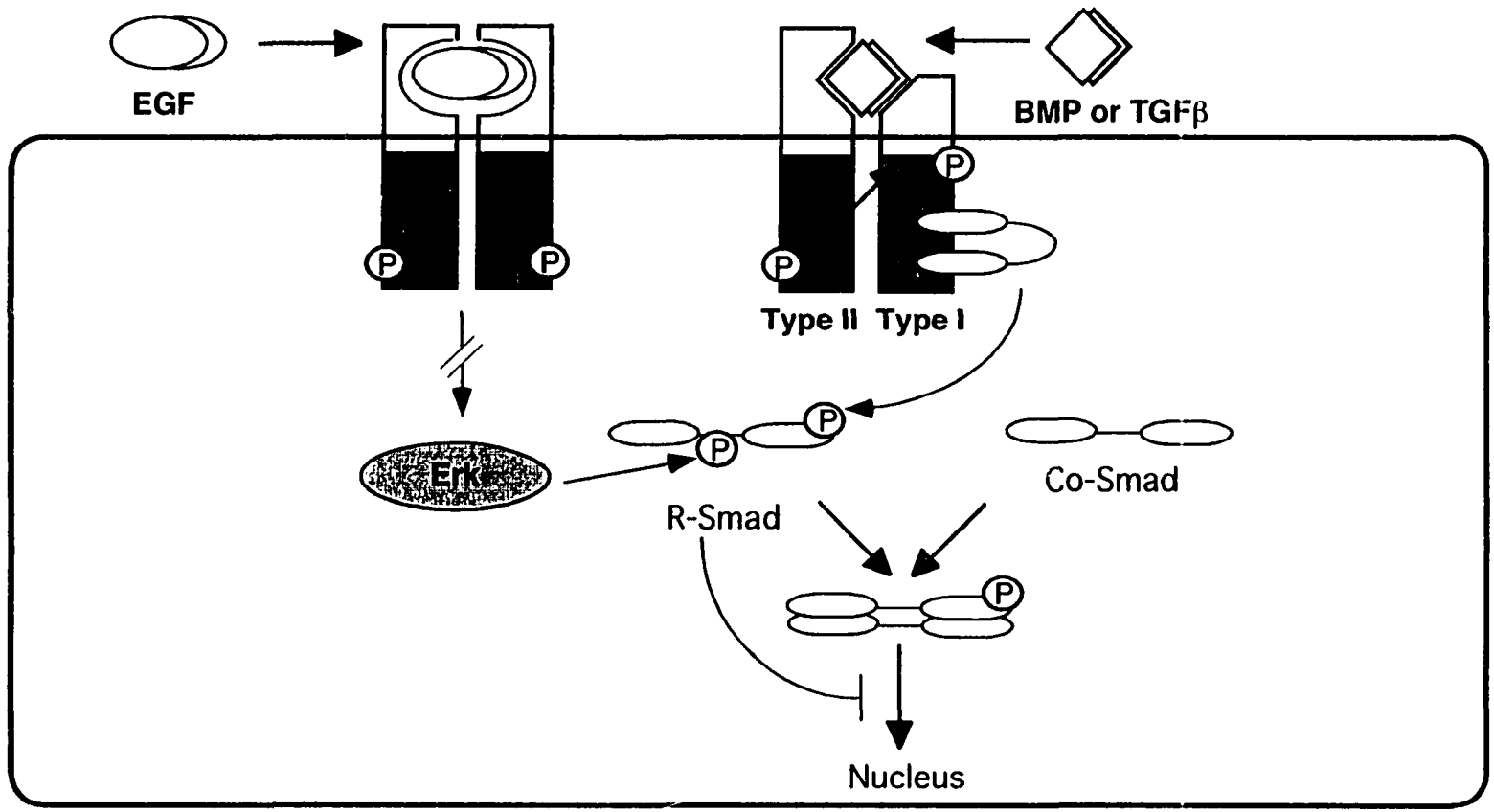

Figure 62. Schematic representation of the regulation of Smad proteins by opposing signaling inputs mediated by EGF and BMP or TGFβ. EGF binding to its receptor initiates a signaling kinase cascade which culminates in the phosphorylation and activation of Erk. Erk in turn phosphorylates the R-Smads in the linker region, thus preventing the R-Smad-Smad4 complex from entering the nucleus.

observed. Therefore, EGF and other mitogens may not be expected to cause extensive inhibition of TGFβ or BMP but instead the cell senses the levels of the various growth factors and determines which response is appropriate. This is supported by reports showing distinctive antagonistic effects between BMP and receptor tyrosine kinase pathways in several developmental systems while other reports indicate they can also function synergistically. As mentioned earlier, dpp and EGF are antagonists in tracheal placode development in *Drosophila* (Wappner et al., 1997). Spatial distribution of digits in chick and mouse limb growth is accomplished by opposing effects of fibroblast growth factor (FGF) and BMP (Niswander and Martin, 1993; Ganan et al., 1996) and antagonistic interactions between FGF and BMP are necessary for tooth formation in mice (Neubuser et al., 1997). Yet activin-like TGFβ family members cooperate with Ras signals in the induction of mesoderm in *Xenopus* (Whitman and Melton, 1992) and Ras and dpp synergize in activating *Ubx* during endoderm formation in *Drosophila* (Szuts et al., 1998). These results indicate that in some biological contexts, antagonism between Smad and Erk may not occur and thus the interplay between Smad signaling and tyrosine kinase cascades is likely to be quite complex.

Oncogenic Ras represses TGFβ signaling

Ras mirrors EGF effects

Parallel experiments involving EGF and Ras demonstrate that oncogenic Ras inhibits TGFβ signaling by the same mechanism as EGF. Oncogenic Ras: 1) induces Smad phosphorylation at even higher levels than EGF; 2) phosphorylates at the S(T)P sites of the linker region in both Smad2 and Smad3; 3) does not effect phosphorylation of the C-terminal SSXS motif; and 4) inhibits Smad2 and Smad3 nuclear accumulation. Results of these Ras-mediated effects on Smad2 and Smad3 are a decrease in TGFβ-activated transcription of responsive genes. Utilizing the A3-Luc construct that responds specifically to activin and TGFβ demonstrates that transfection in a Ras-resistant Smad3(4AP) mutant can rescue cells from Ras-mediated transcriptional inhibition. These results indicate oncogenic Ras inhibits TGFβ signaling by similar means as EGF, differing only in their intensities. EGF signaling is transitory, maximal at 5 minutes and diminishing to basal levels within 2 hrs (Marshall et al., 1995). The rapid and transient activation of Ras by EGF and other mitogens contrasts with the sustained activation of Smad by TGFβ, which is maximal at 1hr and is sustained for several hours. This may limit the degree to which Ras activation interferes with Smad signaling, resulting in conflicting reports of antagonism between the two pathways. Where Ras is activated constitutively, such as Ha-Ras^{V12}, there is

a sustained effect on Smad and thus the effects are more potent and more persistent.

These observations are borne out in EpRas cells, which are stably Ras-transfected EpH4 mammary cells. EpRas has an increased level of Smad2/3 phosphorylation in the absence of TGFβ but do not inhibit TGFβ phosphorylation of Smad2, mimicking results seen in cells transfected with oncogenic Ras. Upon TGFβ addition, EpH4 cells show 95% nuclear translocation of Smad2/3 within 30 minutes of TGFβ exposure, which is sustained for 3 hrs before levels of Smad2/3 drop off. These results are similar to those observed in other TGFβ-responsive cell lines we have tested. In contrast, EpRas cells showed a limited accumulation of Smad2/3 that never reached the amounts seen in EpH4 cells. Thus, oncogenic Ras has a sustained effect on Smad nuclear accumulation and this may explain why Ras is more potent as a TGFβ signaling inhibitor than EGF.

Ras-induced Smad Phosphorylation may play a role in tumor progression

Loss of antiproliferative responses to TGFβ in cancer cell lines led to the belief that inhibition of TGFβ signaling might predispose or cause cancer. The first indication to validate this assumption was the discovery of microsatellite instability of TGFβ receptor type II in colorectal cancer (Markowitz et al., 1995; Markowitz and Roberts,1996). Additionally, TGFβ receptor type I has been found to carry a polymorphism that may contribute to cancer development (Pasche et al., 1998; Pasche et al., 1999). Inactivating mutations in both Smad2 and Smad4

have been found in various human cancers including colorectal, lung and pancreatic carcinomas (Eppert et al., 1996; Hahn et al., 1996; Riggins et al., 1996, reviewed in Hata et al., 1998). Yet, many tumor cells without known mutations in these molecules are refractory to growth inhibition by TGFβ.

Substantial portions of colon and pancreatic carcinomas contain oncogenic Ras mutations (Fearson and Vogelstein, 1990; Kern, 1998) while breast carcinomas often contain Her2 and EGFR amplifications (Clark and Der, 1995). Transfection in oncogenic Ras leads to loss of TGFβ-induced growth arrest and loss of sensitivity to TGFβ (Schwarz et al., 1988; Houck et al., 1989; Valverius et al., 1989; Manning et al., 1991; Filmus et al., 1992; Longstreet et al. 1992, our observations). Therefore, the presence of oncogenic Ras may lead to tumor cells being refractory to TGFβ. The colon carcinoma cell lines of known Ras status that we screened show a correlation between the presence of oncogenic Ras mutations and little to no nuclear accumulation of Smad2/3. EpRas cells that have a stably transfected Ras mimic this effect where EpH4 parental cells have a normal nuclear translocation of Smad2/3 in response to TGFβ. EpRas cells, moreover, have a reduced activity of A3-luc in the presence of TGFβ correlating oncogenic Ras inhibition of Smad nuclear accumulation with decreased transcriptional activity.

We were unable to rescue TGFβ signaling of either EpRas or colon cancer cell lines by using Ras inhibitors because the only inhibitor available is the MEK1 inhibitor PD98059. PD98059 prevents an activating phosphorylation of MEK1 by

Ras. If however, as in our case, an activated Ras constitutively phosphorylates MEK1, PD98059 is unable to function in its capacity as a Ras inhibitor. We were able to rescue EpRas cells by transfection of Smad3(4AP) mutants that cannot be phosphorylated by oncogenic Ras. In an A3-luc assay, EpRas cells containing Smad3(4AP) show the same luciferase activity as EpH4 cells transfected with either Smad3 wild type or Smad3(4AP).

SW480 colon cancer cells contain an activated Ki-Ras oncogene and defects in Smad4 function. When Smad4 is transfected into SW480 cells, some of the TGFβ responses are restored but activity is weak, indicating that the Smad4 mutation is not the sole reason for TGFβ unresponsiveness in SW480 cells (Calonge and Massague, 1999). Furthermore, SW480 cells have decreased Smad3 nuclear accumulation, agreeing with our observations that Smad3 nuclear accumulation is hindered in colon cancer cell lines containing an oncogenic Ras. Cotransfection of Smad3(4AP) with Smad4 led to the recovery of A3-luc as well as various other gene responses. These results corroborate our own suggesting that inhibition of Smad2/3 function by an oncogenic Ras occurs via phosphorylation of SP sites that prevent TGFβ responses and growth inhibition. The inhibitory mechanism described here may allow the development or expansion of tumors due to loss of TGFβ function. Ras repression of Smad nuclear accumulation may be one mechanism by which tumors escape the growth inhibitory effects of TGFβ.

IFNγ represses TGFβ signaling by upregulating Smad7

IFNγ-mediated phosphorylation of Smad3 is not required for IFNγ-induced inhibition

Using the A3-luc reporter construct we demonstrate that IFNγ inhibits TGFβ signaling in U4A cells, which have a stably transfected Jak1. When Jak1 is mutated, this inhibition of TGFβ-induced transcriptional activation by IFNγ is absent, indicating this inhibition requires Jak1 and therefore requires the IFNγ signaling pathway to be intact. These results are not surprising because the antagonism between IFNγ and TGFβ signaling is well documented (Bauvois et al., 1992; McCartney-Frances and Wahl, 1994; Schmitt et al., 1994).

The mechanism by which IFNγ impedes TGFβ signaling must be an early event since this work demonstrates that IFNγ inhibits nuclear accumulation of Smad3 as well as complex formation with Smad4 in U4A/Jak1 cells treated with TGFβ. Since EGF inhibits TGFβ by phosphorylating the linker region of Smad2 and Smad3, it is reasoned that a similar mechanism might be at work in IFNγ inhibition of TGFβ signaling. Indeed, it is shown that IFNγ induces phosphorylation of Smad3 within 15 minutes of addition and this phosphorylation occurs at the SP sites of the linker region. Furthermore, MEK1 is responsible for IFNγ-mediated phosphorylation of Smad3, reminiscent of the results obtained with EGF. However, when a MEK1 inhibitor is added along with IFNγ and TGFβ, it only rescues TGFβ response by a small margin. IFNγ still inhibits TGFβ-induced A3-luc response. Therefore, even though MEK1 inhibitor prevents IFNγ-

induced Smad3 phosphorylation, MEK1 inhibitor does not prevent IFNγ inhibition of TGFβ signaling. There must be another mechanism involved in IFNγ-induced TGFβ inhibition.

IFNγ induces Smad7 upregulation, which inhibits TGFβ signaling

Several observations indicate that IFNγ-induced inhibition of TGFβ is due to increased Smad7 levels in the cell. First, by using affinity-labeled receptors it is demonstrated that Smad3 receptor complexes are decreased in IFNγ treated cells. Since the level of receptors is not affected by the incubation of cells with IFNγ, Smad3 must be prevented from binding to the receptors. Smad7 binding to the TGFβ receptor complex would prevent Smad3 interaction with these receptors (Hayashi et al.1997; Nakao et al., 1997b). Second, IFNγ induces the expression of Smad7 mRNA in U4A/Jak1 cells, within 30 minutes of IFNγ addition. IFNγ does not induce other inhibitors of TGFβ showing specificity for Smad7 upregulation. Third, IFNγ raises the amounts of Smad7-bound receptor complexes, which would explain the decrease in Smad3-bound receptor complexes since Smad7 displaces Smad3 in these complexes (Hayashi et al., 1997; Nakao et al., 1997b; Souchelnytskyi et al., 1998). And finally, as would be expected from inhibiting Smad3-TGFβ receptor complexes, TGFβ does not phosphorylate Smad3 when IFNγ is present. Incubating cells with Smad7 antisense oligonucleotides in the presence of IFNγ reverses this decrease in phosphorylation of Smad3. Thus, those results indicate a mechanism for

modulation of the TGFβ signaling pathway by IFNγ: IFNγ signaling through the Jak1 pathway rapidly increases the expression of the anti-Smad, Smad7, resulting in inhibition of TGFβ-mediated Smad3 phosphorylation and consequently prevents Smad3 nuclear translocation and transcriptional activation of TGFβ–responsive genes (Fig. 63).

EGF has also been shown to increase *Smad7* mRNA expression (Afrakhte et al., 1998), effectively utilizing two mechanisms by which it inhibits TGFβ responses; phosphorylation of the R-Smads in the linker region and Smad7 upregulation. More recently, TNFα and interleukin 1B were shown to induce Smad7 synthesis in their suppression of TGFβ signaling (Bitzer et al., 2000). Using similar techniques, it was demonstrated that TNFα, via NF-κB/RelA, inhibits TGFβ-mediated phosphorylation of Smad2 and subsequently Smad2 nuclear translocation and transcriptional activity. Inhibition of Smad2 is concomitant with upregulation of *Smad7* mRNA levels and association with TGFβ receptor complexes. Using *Smad7* antisense oligonucleotides they were able to release TGFβ-induced transcriptional responses from inhibition by NF-κB/RelA. Thus, antagonists of TGFβ signaling, such as TNFα, use the same mechanism of inhibition of TGFβ action as IFNγ, namely the upregulation of the I-Smad, Smad7. TNFα and other NF-κB inducers are proinflammatory cytokines (Baeuerle and Henkel,1994), contrary to TGFβ, which is a known immunosuppressor (Letterio and Roberts, 1997). Conversely, inducers of NF-κB, such as TNFα, inhibit TGFβ activation of matrix synthesis, apoptosis and hematopoiesis (Oberhammer et al.,

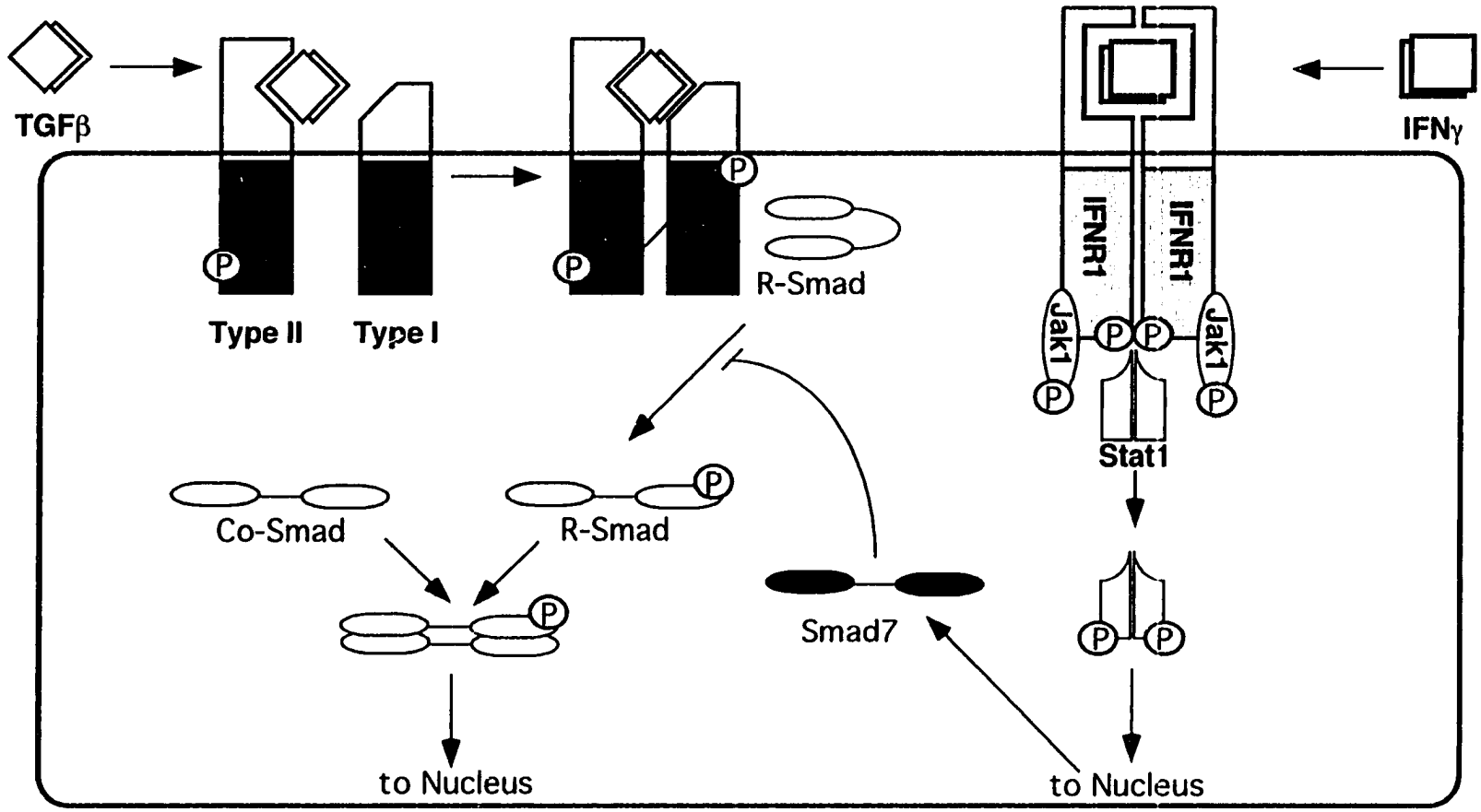

Figure 63.A model of the interactions between the Stat and Smad pathways. IFNγ-mediated activation of Jak1 and Stat1 leads to induction of Smad7, which inhibits the ability of the TGFβ-receptor complex to interact with, and activate, Smad3. As a result, transcriptional responses to TGFβ signaling are inhibited.

1992; Inagaki et al., 1995; Snoeck et al., 1996). Therefore, crosstalk between the two pathways is likely, and by upregulating Smad7 NF-κB-mediated pathways use an elegant method to antagonize TGFβ signaling, a mechanism evidently utilized by several TGFβ antagonists.

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