Identification of three novel Smad binding proteins involved in cell polarity

Dennis R. Warner*, M. Michele Pisano, Emily A. Roberts, Robert M. Greene

University of Louisville Birth Defects Center, Department of Molecular, Cellular, and Craniofacial Biology, University of Louisville School of Dentistry, 501 South Preston Street, Suite 301, Louisville, KY 40292, USA

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Abstract **A yeast two-hybrid screen was utilized to identify novel Smad 3 binding proteins expressed in developing mouse orofacial tissue. Three proteins (Erbin, Par-3, and Dishevelled) were identified that share several similar structural and functional characteristics. Each contains at least one PDZ domain and all have been demonstrated to play a role in the establishment and maintenance of cell polarity. In GST (glutathione *S*-transferase) pull-down assays, Erbin, Par-3, and Dishevelled bound strongly to the isolated MH2 domain of Smad 3, with weaker binding to a full-length Smad 3 protein. Failure of Erbin, Par-3, and Dishevelled to bind to a Smad 3 mutant protein that was missing the MH2 domain confirms that the binding site resides within the MH2 domain. Erbin, Par-3, and Dishevelled also interacted with the MH2 domains of other Smads, suggesting broad Smad binding specificity. Dishevelled and Erbin mutant proteins, in which the PDZ domain was removed, still retained their ability to bind Smad 3, albeit with lower affinity. While transforming growth factor β (TGFβ) has been suggested to alter cell polarity through a Smad-independent mechanism involving activation of members of the RhoA family of GTP binding proteins, the observation that Smads can directly interact with proteins involved in cell polarity, as shown in the present report, suggests an additional means by which TGFβ could alter cell polarity via a Smad-dependent signaling mechanism.**

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1. Introduction

Transforming growth factor β (TGFβ) is a member of a large superfamily of growth and differentiation factors composed of three subfamilies: the TGFβs, activins/inhibins, and bone morphogenetic proteins (reviewed in [1]). The TGFβs elicit a wide variety of biological actions including embryonic morphogenesis, cell proliferation, cell differentiation, apoptosis, and extracellular matrix synthesis [1]. TGFβ exerts its influence on cells by altering patterns of gene expression such as those genes controlling extracellular matrix synthesis (e.g. collagen, collagenase, tenascin, matrix metalloproteinases, and the plasminogen activator inhibitor 1) [2–5] and those genes controlling cell proliferation (e.g. c-myc and JunB) [6,7].

The TGFβ signaling cascade is initiated by binding of TGFβ to a type II receptor-serine/threonine kinase (TβRII) that then heterodimerizes with a type I receptor (TβRI, also a serine/threonine kinase), resulting in phosphorylation of TβRI by TβRII (reviewed in [1]). Activated (phosphorylated) TβRI subsequently recruits and phosphorylates a class of proteins termed Smads. The Smad proteins function as transducers of TGFβ signaling and are categorized in three distinct groups based on function and sequence homology (receptor-regulated, common, and inhibitory). Receptor-regulated Smads (R-Smads), which are substrates for phosphorylation by TGFβ type I receptors, are further divided into those regulated by TGFβ/activin (Smads 2 and 3) and those regulated by bone morphogenetic proteins (Smads 1, 5, and 8). Smad 4 is a 'common' Smad that heterodimerizes with phosphorylated R-Smads. This Smad complex then translocates into the nucleus. Inside the nucleus, the Co-Smad/R-Smad complex binds to specific sequences within the promoters of TGFβ-responsive genes and, depending on the cell type or gene, either stimulates or represses transcription. Transcriptional activation by Smads occurs with the aid, in part, of nuclear coactivators such as CBP and p300 [8]. Conversely, transcriptional repression is effected by interaction with nuclear corepressors such as SnoN and c-Ski [9,10]. Thus, depending on the particular cellular complement of nuclear coactivators and corepressors, the transcriptional outcome of Smad/DNA binding will vary. Although Smads are capable of directly binding DNA, they do so with low affinity [11]. High affinity binding of Smads to DNA requires additional cofactors for the full transcriptional response [12].

TGFβ family members are critical regulators of mammalian orofacial development in that they are expressed in precise spatio-temporal patterns in the developing orofacial region and act specifically through control of cellular proliferation and differentiation in the tissue [13,14]. Mouse knock-out experiments have provided compelling evidence that fusion of the developing secondary palatal shelves requires TGFβ3-induced medial edge epithelial (MEE) transdifferentiation [15], a process wherein MEE cells differentiate into a mesenchymal phenotype via epithelio-mesenchymal transformation (EMT) [16]. The critical role of TGFβ signalling in orofacial develop-

*Corresponding author. Fax: (1)-502-852 8309.
E-mail address: drwarn01@gwise.louisville.edu (D.R. Warner).

Abbreviations: TGFβ, transforming growth factor β; MEE, medial edge epithelia; Dvl, Dishevelled; EMT, epithelio-mesenchymal transformation; TβRI and II, TGFβ receptor types I and II, respectively; PDZ, PSD-95, Discs-large, and ZO-1 domain

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ment and the likelihood that cell- and tissue-specific responses to TGFβ are mediated by cell- and tissue-specific Smad co-factors prompted a search for unique Smad binding proteins expressed in embryonic orofacial tissue. A yeast two-hybrid assay was employed in which the MH2 domain of Smad 3 was used to screen an expression library derived from mouse embryonic orofacial tissue. Three proteins have been identified from this screen that share similar structural and functional characteristics. Dishevelled-1 (Dvl-1), a protein intermediate in the Wnt signaling pathway [17], Par-3, a protein originally identified in *Caenorhabditis elegans* mutants exhibiting defects in partitioning (cell polarity) [18], and Erbin, a recently identified protein that binds to the Erbb2 receptor [19]. Dvl-1, Par-3, and Erbin are all PDZ (PSD-95, Discs-large, and ZO-1) domain containing proteins that have been implicated in establishment and maintenance of cell polarity.

The present report provides a preliminary characterization of the interaction between Smad 3 and these three novel Smad binding proteins (Dvl-1, Erbin, and Par-3). To our knowledge, this is the first report of cross-talk between the TGFβ and Wnt signaling pathways at the level of Dvl and the first report of an interaction between Smads and cell polarity proteins (Par-3 and Erbin).

2. Materials and methods

2.1. Animals

ICR mice (Harlan, Indianapolis, IN), were maintained at a temperature of 22°C with an alternating light/dark cycle and were provided access to food and water ad libitum. Mature male and female mice were mated overnight and the presence of a vaginal plug the following morning was taken as evidence of mating (gestation day 0). Pregnant mice were euthanized on days 11–13 of gestation, a critical stage of murine orofacial development. Embryos were removed from pregnant dams, embryonic maxillofacial tissue was dissected in sterile, cold phosphate-buffered saline, and RNA was extracted from embryonic tissue according to established protocols.

2.2. Yeast two-hybrid embryonic orofacial tissue expression library

RNA was isolated from mouse embryonic maxillary tissue harvested from embryos collected on days 11–13 of gestation. The expression library was prepared by Research Genetics, Inc. (Huntsville, AL) and has an average insert size of 2.0 kb. In order to generate the library, embryonic orofacial cDNAs were inserted into the pGADT7 vector (Clontech, Palo Alto, CA) which previously had been modified to remove the existing *Not*I site at position 4520. A linker encoding a *Not*I site flanked by *Bam*HI sites was inserted into the unique *Bam*HI site within the multi-cloning sequence of pGADT7. Embryonic orofacial cDNAs were then inserted into the *Eco*RI–*Not*I site of the modified pGADT7 vector to generate the final expression library.

2.3. Construction of fusion proteins with Smad 3 or Smad 3 MH2 domain and the Gal 4 DNA binding domain

Full-length human Smad 3 cDNA (in pcDNA3, Invitrogen, Carlsbad, CA) was obtained from Dr. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). To create a Gal4 DNA binding domain–Smad 3 fusion protein ('bait'), the Smad 3 cDNA was released by digestion with *Eco*RI and *Xho*I. The resulting 1.4 kb Smad 3 cDNA was ligated into *Eco*RI/*Sal*I-digested pGBKT7 (Clontech) downstream of the DNA binding domain of Gal4. To generate an additional 'bait' construct, with the MH2 domain of Smad 3, polymerase chain reaction (PCR) was used to amplify the portion of the cDNA encoding amino acid residues 239–424. An *Eco*RI site was inserted at the 5′-end of the fragment, and the existing *Xho*I site at the 3′-end was maintained. The limits of the MH2 domain were chosen based on the crystal structure of the Smad 4 MH2 domain [11]. The sequence of the forward PCR primer was 5′-CGGAATTCGCCTTCTGGTGCTCCATCTCC-3′ and the reverse primer was 5′-CATGCTCGAGCTAAGACACACTGG-3′. The PCR product was digested with *Eco*RI and *Xho*I and inserted into pGBKT7 digested with *Eco*RI and *Sal*I (downstream of the Gal4 activation domain). All constructs were sequenced to verify integrity of the constructs. Furthermore, synthesis of either full-length Smad 3 protein or the 24 kDa MH2 domain of the Smad 3 protein was tested via in vitro transcription/translation using the TNT T7 coupled transcription/translation system from Promega (Madison, WI) and [^{35}S]L-methionine (Perkin-Elmer Lifesciences, Boston, MA).

2.4. Yeast two-hybrid screen

pGBKT7-Smad 3 MH2 was cotransformed into *Saccharomyces cerevisiae*, strain AH109, along with the embryonic orofacial tissue expression library and plated onto yeast medium lacking histidine, leucine, tryptophan, adenine, and supplemented with 2.5 mM 3-amino-1,2,4,-triazole. The resulting transformants (approximately 5×10^5) were replica-plated to the same type of plates supplemented with 20 μg/ml X-α-gal. Blue colonies with the His$^+$/Leu$^+$/Trp$^+$/Ade$^+$ phenotype were cultured and the plasmids were isolated using standard protocols [20]. Clones were re-introduced into yeast strain AH109 along with either empty pGBKT7, pGBKT7-Smad 3 MH2, pGBKT7-Smad 3 (full-length) or pGBKT7-lamin C (negative control) to verify the Smad:clone interaction. Clones maintaining the His$^+$/Leu$^+$/Trp$^+$/Ade$^+$/X-α-gal$^+$ phenotype were sequenced. Individual clones were then transcribed and translated in vitro to insure proper synthesis of each protein and determine the encoded protein's molecular weight.

2.5. Glutathione S-transferase (GST) pull-down assay

GST-fusion protein constructs for GST pull-down assays included those for: Smad 1, Smad 1 MH2, Smad 2, Smad 2 MH2, Smad 3, Smad 3 MH2, Smad 4, Smad 4 MH2, Smad 7, and Smad 7 MH2. To generate GST-fusion constructs for GST pull-down assays with full-length Smad 3 or Smad 3 MH2, each cDNA was subcloned from pGBKT7 into pGEX-5X-1 (Amersham Biosciences, Piscataway, NJ). pGEX2TK-human Smad 1 was provided by Dr. X. Lin (Baylor University, Houston, TX), pGEX4T2-mouse Smad 2 and pGEX4T2-human Smad 4 were provided by Dr. Y. Chen (Indiana University, Indianapolis, IN) and pGEX4T-1-Smad 3 ΔMH2 was obtained from Dr. M. Kato (Japanese Foundation for Cancer Research, Tokyo, Japan). pGEX-5X-1 Smads 1-, 2-, and 4-MH2 domains were generated by PCR with primers specific for the MH2 domains, and utilizing full-length cDNA's as the template. The MH2 domains encompass amino acid residues 269–465 (Smad 1), 272–467 (Smad 2) and 321–552 (Smad 4). pGEX-5X-1 *Xenopus* Smad 7 full-length and Smad 7 MH2 domain (amino acid residues 214–390, based on sequence homology with the MH2 domain of Smads 1–5 and 8) were constructed by performing PCR on pBluescript II SK containing full-length *Xenopus* Smad 7, provided by Dr. H. Brivanlou (Rockefeller University, New York, NY). All constructs were verified by DNA sequencing. Each was transformed into *Escherichia coli* BL21, and GST-fusion protein expression was induced with 0.3 mM isopropyl thio-β-D-galactopyranoside for 6 h. Preparation of cleared lysates and purification of the fusion proteins were as described [20]. Approximately 1 μg of GST-fusion protein was mixed with 5 μl in vitro translated, [^{35}S]-labeled Dvl-1, Par-3, or Erbin protein in GST pull-down buffer (20 mM Tris, [pH 7.5], 150 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, 10% glycerol, 1 mM EDTA, 2.5 mM $MgCl_2$, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The reaction was incubated for 2 h at 4°C with rotation, and the sepharose beads washed four times with 0.4 ml GST pull-down buffer. Bound protein was released by the addition of 2× Laemmli sample buffer [21] followed by incubation for 5 min at 95°C. The eluted sample was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gels were fixed with 50% methanol:10% acetic acid and exposed to Kodak X-Omat film for 2 days at −80°C. Following autoradiography, each gel was stained with Coomassie blue to determine loading efficiency for each sample.

2.6. α-Galactosidase assay

Secreted α-galactosidase was measured from log-phase cultures of yeast cotransformed with Smad 3 MH2 and Dvl-1 or Erbin deletion mutants. Hydrolysis of *p*-nitrophenyl α-D-galactopyranoside was determined by spectrophotometrically measuring released *p*-nitrophenol (lambda max = 410 nm) according to procedures described by Clontech (Palo Alto, CA).

3. Results

3.1. Yeast two-hybrid screen for Smad binding proteins

In order to contribute to a better understanding of TGFβ signaling mechanisms in mammalian orofacial development, a yeast two-hybrid screen of an expression library generated from RNA isolated from the orofacial region of gestational days 11–13 embryos was performed using the MH2 domain of the TGFβ-responsive Smad 3. Numerous Smad 3-interacting proteins were identified from a screen of approximately 5×10^5 yeast transformants. Three of these binding proteins are described in this report: (1) Dvl-1, an intermediate in the Wnt signaling pathway [17]; (2) Erbin, a recently reported protein that interacts with the Erbb2 receptor [19], and (3) Par-3, a protein which, when mutated, confers a partitioning defect in *C. elegans* [27]. These three proteins have been grouped in the present report based on the presence of a common structural motif and known function. All of these proteins have at least one PDZ domain and have been demonstrated to function in controlling cell polarity and/or asymmetric cell division. PDZ domains are modular domains of approximately 80 amino acids that mediate protein–protein interactions, generally those involved in targeting proteins to specific locations within the cell (reviewed in [22]). These domains were originally identified in PSD-95, Discs-large, and ZO-1, hence the acronym, PDZ.

To demonstrate specificity of binding, plasmid was isolated from each positive two-hybrid clone and introduced back into *S. cerevisiae* along with either full-length Smad 3, Smad 3 MH2 domain, or lamin C fused to the DNA binding domain of Gal 4, or empty vector (Gal 4 DNA binding domain alone). Neither Smad 3 nor the MH2 domain of Smad 3 resulted in activation of the reporter genes (data not shown). Cotransformed yeast were plated onto high-stringency medium (Fig. 1). Growth occurred only in the presence of either full-length Smad 3 or the MH2 domain of Smad 3, demonstrating that Erbin, Par-3, and Dvl-1 all interacted with both full-length Smad 3 and the isolated MH2 domain in this assay.

Each cDNA isolated for these three two-hybrid clones encoded truncated proteins missing their amino termini. The limits of the clones and the positions of conserved domains are illustrated in Fig. 2. The clones for both Erbin and Dvl-1 contained either full or partial PDZ domains, respectively. The Par-3 clone was missing a PDZ domain altogether. Full-length Erbin contains, in addition to the PDZ domain, a leucine-rich region (LRR) at the amino terminus. Such LRR domains have been suggested to mediate protein–protein in-

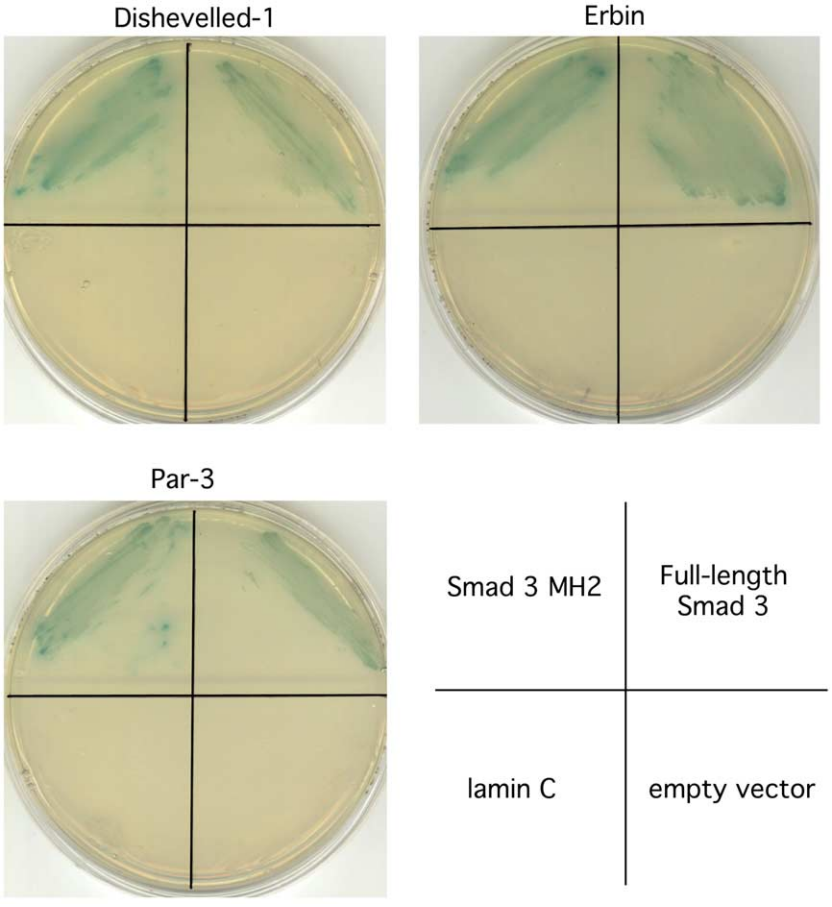

Fig. 1. Interaction between Smad 3 and Erbin, Par-3, and Dvl-1 in a yeast two-hybrid assay. Growth of transformed *S. cerevisiae* on high-stringency plates (see Section 2) demonstrating an interaction between either Smad 3 MH2 or full-length Smad 3 and Erbin, Dvl-1, or Par-3. pGBKT7-Smad 3 MH2, pGBKT7-h Smad 3 (full-length), pGBKT7-lamin C, or empty pGBKT7 were cotransformed with pGADT7-Erbin, pGADT7-Dvl-1, or pGADT7-Par-3 into *S. cerevisiae* AH109 and initially plated onto low-stringency plates (see Section 2). Colonies were then re-streaked onto high-stringency plates in the presence of X-α-gal. Note growth and blue color when Erbin, Dvl-1, or Par-3 were cotransformed with either full-length Smad 3 or its MH2 domain but not with empty pGADT7 or pGADT7-lamin C.

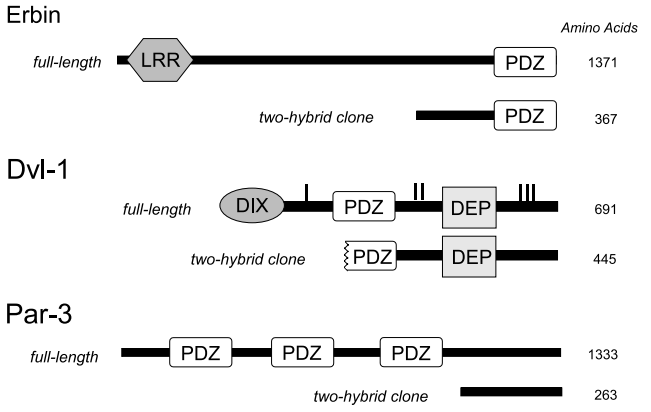

Fig. 2. Schematic illustrating the position of the clones isolated in the yeast two-hybrid screen in relation to their full-length counterparts. The domain organization of full-length Erbin, Par-3, and Dvl-1 was obtained from primary references [18,19,24]. For the purpose of the present study, the intervening regions of Dvl-1 are arbitrarily labelled I, II, and III.

teractions [23]. The Dvl clone corresponded to Dvl isoform 1 [24]. In addition to a PDZ domain, full-length Dvl-1 contains domains found in other proteins. The DIX domain of Dvl is similar to that found in Axin which mediates formation of a protein–protein complex between Dvl isoforms and Axin [25]. The DEP domain of Dvl shares homology to domains found in proteins such as the product of the *C. elegans* egl-10 gene and pleckstrin [26]. The Par-3 clone corresponded to the reported 180 kDa form and contained no discernable domains [18].

3.2. Erbin, Par-3, and Dvl-1 preferentially interact with the MH2 domains of several Smads

To confirm the Smad–protein interaction in vitro and to determine if Erbin, Par-3, and Dvl-1 bind other Smad proteins, Smad proteins from each class (receptor-regulated, common, and inhibitory) were expressed in *E. coli* as a fusion protein with GST and the fusion proteins were purified on glutathione beads. In vitro translated, [^{35}S]methionine-labeled Erbin, Par-3, and Dvl-1 were individually mixed with purified GST–Smads and 'pulled-down' with glutathione–agarose beads. All three proteins interacted with the isolated MH2 domain of Smad 3 (Fig. 3) which is consistent with the data from the yeast two-hybrid screen (Fig. 1). In addition to Smad 3 MH2, Erbin bound to the MH2 domains of Smads 2, 4 and 7 and with weaker binding to the MH2 domain of Smad 1. Erbin bound poorly to full-length versions of Smads 1, 4, and 7. Dvl-1 exhibited a pattern of binding similar to Erbin with the exception that binding to full-length Smad 7 was greater than with the isolated Smad 7 MH2 domain. Par-3 bound poorly to all full-length Smads, with the notable exception of Smad 7, in which binding was more efficient than the isolated MH2 domain. In contrast to both Erbin and Dvl-1, Par-3 bound weakly to Smads 1 and 2 (either full-length and MH2 domain). These data demonstrate that Erbin, Par-3, and Dvl-1 exhibit broad specificity of binding to Smads, with a preference for the Smad MH2 domain (with the obvious exception of Smad 7). All three of these proteins bound inefficiently to a mutant of Smad 3 in which the MH2 domain was deleted (Smad 3 ΔMH2, Fig. 3), demonstrating that the site of interaction lies within the MH2 domain. Additionally, no interaction was observed with GST alone.

3.3. PDZ domains do not bind Smads

To determine if the PDZ domains of Erbin and Dvl-1 confer the ability to interact with Smad 3, deletion mutants were constructed to remove the PDZ domains of these two proteins. Each mutant cDNA was transformed into yeast along with the MH2 domain of Smad 3. The strength of interaction was tested by measuring the secretion of α-galactosidase, which is directly proportional to the strength of the protein–Smad 3 interaction. As shown in Table 1, deletion of the PDZ domain reduced binding to Smad 3 by approximately 50% for both Dvl-1 (Dvl-1 ΔPDZ) and Erbin (Erbin ΔPDZ). An isolated PDZ domain of Erbin (Erbin PDZ domain) failed to bind Smad 3 MH2. These data demonstrate that the PDZ domains of Dvl-1 and Erbin are not solely responsible for interaction with Smad 3 MH2. Dvl-1 also contained an additional domain commonly found in GTP binding proteins termed the DEP domain. Deletion of this domain (Dvl-1 ΔDEP), reduced Smad 3 binding to one-third that of the

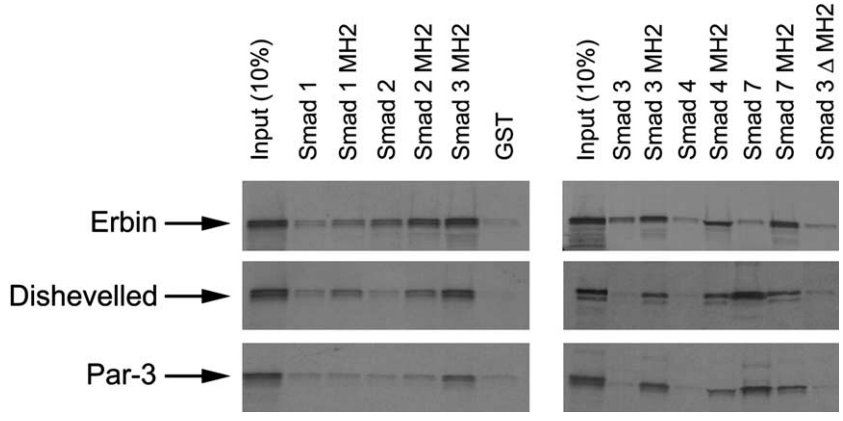

Fig. 3. GST pull-down assay between Erbin, Par-3, or Dvl-1 and various Smads. GST–Smad fusion proteins were expressed and purified from *E. coli*. Approximately 1 μg GST–Smad fusion protein was mixed with [^{35}S]methionine-labelled, in vitro translated Erbin, Par-3, or Dvl-1 (truncated versions isolated during the two-hybrid screen), precipitated with glutathione–agarose and analyzed by SDS–PAGE and autoradiography. The autoradiograms presented are representative of three independent experiments and confirm binding of Par-3, Erbin, and Dvl-1 to Smad 3 MH2 and identifies binding to additional Smads. 'Input' lane demonstrates the signal from 10% of the amount of in vitro translated protein present in each sample.

Table 1
Deletion mapping of Dvl and Erbin Smad 3 binding domains

cDNA	α-Galactosidase activity (% of unmodified protein)
Dvl-1	100*
Dvl-1 ΔPDZ	43.1 ± 4.1
Dvl-1 ΔDEP	32.1 ± 3.0
Dvl-1 ΔII	6.6 ± 1.5
Dvl-1 ΔDEP/III	22.0 ± 3.3
Dvl-1 ΔPDZ/II	15.3 ± 1.2
Erbin	100**
Erbin ΔPDZ	50.9 ± 3.0
Erbin PDZ domain	0.9 ± 0.9

Dvl-1 and Erbin deletion constructs in pGADT7 were introduced into *S. cerevisiae* AH109 along with the cDNA for the MH2 domain of Smad 3 (in pGBKT7), plated onto SD/-Leu/-Trp plates, and incubated for 3 days. Transformants were used to establish log-phase liquid cultures, from which the levels of α-galactosidase secreted into the media were determined by measuring hydrolysis of PNP-α-gal. The data, representative of three independent experiments, are reported as the mean ± range of replicate determinations. Data are expressed as percentage of α-galactosidase activity compared to the unmodified protein (which is set to 100%), and are normalized to cell culture density. Background activity, which was determined by cotransforming each test plasmid with lamin C, was subtracted for final presentation of the data and ranged from 0.1 to 1.3 mU/ml, for Dvl-1 and Erbin, respectively. The absolute values for α-galactosidase activity for unmodified proteins were: *15.0 ± 0.7 and **10.7 ± 0.5 mU/ml. 'Unmodified protein' refers to the clone isolated in the two-hybrid screen.

full-length Dvl-1 clone, demonstrating that it plays a role in Smad 3 binding. Finally, mutation of the sequences between the PDZ and DEP domains of Dvl-1 and the carboxyl-terminal 195 amino acids was carried out to determine their function in Smad 3 binding. When the PDZ and DEP domains were moved next to each other (Dvl-1 ΔII), only 6.6% of the level of binding to Smad 3 obtained from the unmodified Dvl-1 was detected, suggesting an important role for this intervening sequence. When the Dvl-1 clone was split in half (Dvl-1 ΔDEP/III and Dvl-1 ΔPDZ/II), each half resulted in similar α-galactosidase activity, (15–20%) of maximal activity. These data suggest that both the PDZ and DEP domains provide binding platforms for Smad 3 and that proper spacing between the two is crucial for efficient binding. For Dvl-1, deleting any series of amino acids significantly decreased binding to Smad 3 MH2. The greatest effect on Dvl-1 binding to Smad 3 MH2 occurred when the sequence between the PDZ and DEP domains was removed (domain II). This suggests that either domain II confers Smad 3 binding or that the spacing between PDZ and DEP domains is critical.

4. Discussion

TGFβ signaling through Smads is critical for a variety of cellular and developmental processes (reviewed in [1]). To further our understanding of TGFβ-directed, Smad-dependent signaling in developing orofacial tissue, a yeast two-hybrid screen was employed to identify unique Smad binding proteins. In the present report, three novel proteins that interact with Smad 3 are identified. These Smad binding proteins possess a common structural motif in that each has at least one PDZ domain. Moreover, they share a common function in that each has been shown to be involved in establishing and/or maintaining cell polarity. PDZ domains are small, modular protein domains of 80–90 amino acids that bind to sequences typically found within the carboxyl terminus of target proteins (reviewed in [22]). They provide a platform for the assembly of multi-protein complexes that are targeted to specific subcellular locations. PDZ domains were first described in PSD-95, the *Drosophila* septate junction protein, Discs-large, and the mammalian tight junction protein, ZO-1 (thus, the abbreviation, PDZ). PDZ domain-containing proteins have been implicated in the establishment of cell polarity and the asymmetric distribution of cellular components found in certain cells with specialized functions. Examples include mammalian epithelial cells with apico-basolateral polarity and the axonic-dendritic specialization of neuronal cells. In development, cell polarity can lead to asymmetric cell division in which cell fate determinants are unequally distributed to daughter cells. As such, cell polarity serves as an important mechanism for generating cell fate diversity. Insight into the phenomenon of cell polarity has been gained predominately through the study of several model systems, particularly the zygote of *C. elegans*, the germline cyst of *Drosophila*, and mammalian epithelial cells.

In *C. elegans*, the major genes that control cell polarity are the Par genes, so-called because mutations in these genes lead to partitioning defects in the early zygote [27]. Several of the Par proteins contain PDZ domains. Par-3 and Par-6 contain three and one PDZ domains, respectively. In *C. elegans*, Par-3 was demonstrated to be the nucleation center for a complex that includes Par-6 and an atypical protein kinase (aPKC) [18]. This Par-3/Par-6/aPKC complex determines the position of the anterior portion of the zygote. Likewise, in mammalian epithelial cells, a homologous tripartate complex is found in tight junctions localized to the apical cellular surface [28]. In *Drosophila* epithelial and neural precursors (where the Par-3 homologue is called Bazooka), a similar complex is involved in determining apico-basolateral polarity of epithelial cells and the apical crescent of dividing neuroblasts [29,30]. The involvement of this tripartate protein complex in controlling cell polarity in diverse organisms suggests a mechanism conserved during evolution. Par-3 binds to Par-6 via a PDZ–PDZ domain-mediated interaction [18]. Recently, additional components of the Par-3/Par-6/aPKC complex have been identified. The Rho family members Cdc42 and Rac1 have been found to bind the Par-3/Par-6/aPKC complex through specific interactions with Par-6 [31]. Since only the GTP form binds efficiently to Par-6, it has been suggested that the activity of the complex is regulated through GTP binding. The involvement of Cdc42 and Rac1 in cytoskeletal rearrangement, a process crucial to asymmetric cell division, has been demonstrated previously (reviewed in [32]).

In *Drosophila* epithelia, apical localization of the Par-3/Par-6/aPKC tripartate complex depends upon three additional proteins: the LAP (leucine-rich region and PDZ domain) protein Scribble, Discs-large (Dlg), and Lethal Giant Larvae [31]. In *C. elegans*, cell polarity requires the LAP protein LET-413 [33]. LAP proteins are a recently described class that is characterized by an amino-terminal LRR and one to four PDZ domains. This class of proteins includes LET-413, Scribble, Densin-180, Lano (Lano is an exception and does not contain a PDZ domain), and Erbin. Erbin is the mammalian homologue of *C. elegans* LET-413. Erbin is localized to the basolateral surface of epithelial cells, binds the Erbb2 receptor and targets it to this region of the cell [19]. LAP proteins also have been implicated in controlling the position of adherens junc-

tions. Erbin has several interaction partners, including proteins that bind the cytoskeleton and desmosomes [34].

In *Drosophila*, a different type of polarity, planar cell polarity or tissue polarity, controls the development of sensory bristles. Planar cell polarity is controlled in part by products of the genes frizzled and Dvl, both members of the Wnt signaling pathway (reviewed in [35]). Frizzled is the receptor for the Wnt ligand and Dvl serves as the control point to direct Wnt signals either: (1) to the canonical pathway leading to β-catenin accumulation and regulation of gene expression through the TCF/LEF family of transcription factors or (2) to the planar cell polarity pathway, mediated through the Jun N-terminal kinase (JNK) [36]. Distinct domains in Dvl control each of these processes. The DIX and PDZ domains control the canonical pathway while the DEP domain is responsible for mediating activation of the JNK pathway [36].

The data presented in the current report demonstrate that Smad 3 binds to three proteins with well-documented roles in cell polarity suggesting that TGFβ-activated Smads are involved in the establishment of cell polarity. Although TGFβ has not been previously shown to be directly involved in cell polarity, it has been linked to promotion of EMT, a process characterized by loss of cell polarity, delocalization of E-cadherin from cell junctions, and the assumption of a more spindle-shaped morphology [37–39]. EMT is an essential process in early embryonic development when large-scale tissue reorganization occurs [40]. In addition, TGFβ has been demonstrated to alter the cytoskeleton through a process dependent on Cdc42 and RhoA [41]. Within the orofacial region, differentiation of the MEE cells via EMT occurs subsequent to fusion of the secondary palatal shelves and has been shown to be regulated by TGFβ [15]. The role of Erbin, Par-3, or Dvl-1 in TGFβ-mediated palatal fusion and epithelial transformation is at present unknown.

Erbin and Par-3 are found in a broad distribution pattern in adult tissues, with little information on their expression during development [42,43]. However, Dvl-1 is expressed throughout both adult and embryonic tissues [44]. We have recently determined that Dvl-1 and Erbin are expressed in developing palatal and maxillary tissue in the embryonic mouse (data not shown). Whether if the interaction between Smads and Erbin, Par-3, or Dvl-1 is specific to craniofacial tissue or is a more general phenomenon awaits further study.

Comparison of Erbin, Par-3, and Dvl-1 revealed no obvious sequence similarity that would point to a Smad binding motif. It is possible, however, that each possesses a similar tertiary structure in the absence of primary sequence conservation. The unique ability of Smads to bind to these three unique proteins (Erbin, Par-3, and Dvl-1), which share structural and functional characteristics, suggests a direct role for Smads in Erbin-, Par-3-, and Dvl-1-mediated biological processes (including, but not limited to, cell polarity). Since other classes of Smads (e.g. the common Smad, Smad 4 and the inhibitory Smad, Smad 7) also bind to these proteins, multiple levels of regulation and crosstalk may exist between the Smad signaling pathway and pathways involving Erbin, Par-3, and Dvl-1.

It is interesting that Erbin, Par-3, and Dvl-1 have higher affinity for the isolated MH2 domain, with the exception of Smad7, in the case for Par-3 and Dvl-1. In the basal state, the current model of Smad activation holds that the MH1 and MH2 domains mutually inhibit one another and receptor-mediated phosphorylation of the Smad C-terminus relieves this inhibition. We propose, therefore, that exposure of the MH2 domain through Smad activation is required for interaction with Erbin, Par-3, and Dvl-1. Although this may be the case for Smads 1–4, full-length Smad 7 bound more strongly to Par-3 and Dvl-1. The MH2 domain of Smad 7 is based upon sequence homology to Smads 1–5, all of which have a conserved MH2 domain. Thus, either additional structures within Smad 7 (i.e. outside the MH2 domain) may be important for binding to Dvl-1 and Par-3, or the structure of the Smad 7 MH2 domain is altered by the presence of the amino-terminal region making it a better binding partner than the MH2 domain alone.

Additional in vivo cellular analyses, currently underway in our laboratory, will provide insight into the role and biological significance of the interaction between Smads and Erbin, Par-3, and Dvl-1 in embryonic orofacial development.

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