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A secreted Frizzled related protein, FrzA, selectively associates with Wnt-1 protein and regulates Wnt-1 signaling

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SUMMARY

The *Wnt* gene family encodes proteins that serve key roles in differentiation and development. Wnt proteins interact with seven transmembrane receptors of the Frizzled family and activate a signaling pathway leading to the nucleus. A primary biochemical effect of Wnt-1 signaling is the stabilization of cytoplasmic β-catenin which, in association with transcription factors of the Lef/tcf family, regulates gene expression. The recent identification of a new class of secreted proteins with similarity to the extracellular, ligand-binding domain of Frizzled proteins, soluble Frizzled related proteins (sFRP), suggested that additional mechanisms could regulate Wnt signaling. Here we demonstrate that FrzA, a sFRP that is highly expressed in vascular endothelium and a variety of epithelium, specifically binds to Wnt-1 protein, but not Wnt-5a protein, and modulates Wnt-1 signaling. FrzA associated with Wnt-1 either when expressed in the same cell or when soluble FrzA was incubated with Wnt-1-expressing cells. FrzA efficiently inhibited the Wnt-1 mediated increase in cytoplasmic β-catenin levels as well as the Wnt-1 induction of transcription from a Lef/tcf reporter gene. The effects of FrzA on β-catenin levels could be demonstrated when co-expressed with Wnt-1 or when individual cells expressing FrzA and Wnt-1 were co-cultured. These data demonstrate the existence of a negative regulatory mechanism mediated by the selective binding of FrzA to Wnt-1 protein.

Key words: FrzA, Wnt-1, β-Catenin

INTRODUCTION

The mammalian *Wnt-1* gene encodes a secreted glycoprotein that participates in normal embryonic development and tissue differentiation and, when inappropriately expressed, contributes to mouse mammary tumorigenesis (Cadigan and Nusse, 1997; Nusse and Varmus, 1992). *Wingless* (wg), the *Drosophila* ortholog of *Wnt-1*, also controls key events in segmental pattern formation during embryogenesis and regulates proliferation in some adult tissues (Klingensmith and Nusse, 1994; Phillips and Whittle, 1993; Siegfried and Perrimon, 1994; Skaer and Martinez Arias, 1992).

The highly conserved *Wnt* gene family comprises at least 18 members and despite extensive similarities in amino acid sequence and biochemical properties between Wnt proteins they can mediate distinctly different biological effects, as measured in defined test systems such as assays for mammalian cell transformation or ability to induce axis duplication upon expression in *Xenopus* oocytes (Cadigan and Nusse, 1997; McMahon and Moon, 1989; Olson et al., 1991; Shimizu et al., 1997; Wong et al., 1994). Based on these functional differences, Wnt-1 defines the prototype member of one class whereas Wnt-5a defines the other. At least 8 Wnt receptor family genes have been identified to date (Wang et al., 1996). These genes, called *Frizzled*, encode seven membrane spanning proteins with characteristic cysteine-rich, extracellular domains (CRD) and a short cytoplasmic tail (Perrimon, 1996). The CRD of Frizzled receptors is required for binding to Wnt proteins (Bhanot et al., 1996; Lin et al., 1997). The specificity of different Wnt proteins for the various Frizzled family members has not been described but specific interactions at this level are likely to account for the observed differences in Wnt biological activities.

Genetic and biochemical studies in model systems from a variety of species have elucidated several components of a Wnt/wg activated signal transduction pathway (Cox and Peifer, 1998; Miller and Moon, 1996; Willert and Nusse, 1998). Together these studies indicate that following receptor binding, a cytoplasmic protein, called dishevelled, is phosphorylated and translocated to the plasma membrane. This is followed by a decrease in activity of a serine-threonine kinase, glycogen synthase kinase 3 (GSK3/ZW3), and leads to an increased accumulation of armadillo/catenin proteins. In mammalian cells, there are two armadillo family members, β-catenin and plakoglobin (γ-catenin), that are regulated by Wnt-1 signaling (Bradley et al., 1993; Hinck et al., 1994; Papkoff et al., 1996).

Catenins were originally identified as cytoplasmic proteins that associate with the cadherin cell to cell adhesion molecules and regulate their function (Ozawa et al., 1989; Peifer et al., 1992; Takeichi, 1991). β- and γ-catenin protein degradation

occurs via a ubiquitin-mediated process which is modulated by the tumor suppressor protein, adenomatous polyposis coli (APC), and inhibited by Wnt signaling (Aberle et al., 1997; Orford et al., 1997; Polakis, 1997; Willert and Nusse, 1998). Upon stabilization, β-catenin associates with HMG box transcription factors of the Lef/tcf family, enters the nucleus and regulates transcription (Bienz, 1998; Clevers and van de Wetering, 1997; Nusse, 1997), presumably leading to the demonstrated effects of Wnt proteins on proliferation and differentiation.

The identification of a growing family of secreted proteins with extensive homology to the CRD of Frizzled family proteins (sFRPs) adds further complexity to the Wnt signaling system (Moon et al., 1997; Zorn, 1997). sFRPs are expressed during embryogenesis and in many adult tissues in a pattern that partially overlaps with that of *Wnt* genes (Finch et al., 1997; Hoang et al., 1998; Mayr et al., 1997; Melkonyan et al., 1997; Rattner et al., 1997). The specific functions of sFRPs are not known but since they are secreted and lack a transmembrane sequence they are presumed to bind Wnt proteins and antagonize their effects. One of the sFRPs, Frzb-1, was identified in a screen for proteins that regulate bone formation (Hoang et al., 1996). Frzb-1, can bind to Wnt-1, *Xenopus* Wnt-8 (XWnt-8) and *Drosophila* wg and can antagonize Wnt-1 and XWnt-8 induced axial duplications and gene expression in *Xenopus* assay systems (Leyns et al., 1997; Lin et al., 1997; Mayr et al., 1997; Wang et al., 1997a,b). Several sFRP family members were also identified in a screen for secreted regulators of apoptosis using a tissue culture model (Melkonyan et al., 1997).

FrzA is a sFRP family member that was identified in a screen for genes that are differentially expressed in bovine endothelium (Xu et al., 1998). FrzA is highly expressed in a variety of embryonic and adult tissues including the brain (neuronal cells), lung and kidney (epithelial cells), with particularly abundant expression in aortic endothelium (Finch et al., 1997; Rattner et al., 1997; Xu et al., 1998). FrzA can bind to *Drosophila* wg protein and antagonize secondary embryonic axes induced in *Xenopus* by XWnt-8 and Wnt-2 (Xu et al., 1998). The selective binding of FrzA to specific mammalian Wnt proteins has not yet been defined nor have the specific functions of FrzA in modulating signal transduction been elucidated. Here we demonstrate that FrzA can directly bind to Wnt-1 but not to Wnt-5a protein. We further show that FrzA can inhibit the activities of Wnt-1 in mammalian cells by down-modulating the Wnt-1 dependent increase in β-catenin levels and induction of transcription from a Lef/tcf-dependent reporter gene.

MATERIALS AND METHODS

Cell lines antisera and vectors

L cells were obtained from ATCC. Standard CMV promoter-based expression plasmids for Wnt-1, Wnt-5a and FrzA cDNAs (Smolich et al., 1993; Xu et al., 1998) were constructed using vectors from Invitrogen. A hemagglutinin (HA) tag was added to the C terminus of Wnt-1 and Wnt-5a and a Flag tag was appended to the C terminus of FrzA. A CMV-based eGFP expression vector was obtained from Clontech. A Lef/tcf reporter construct was previously constructed and characterized (Papkoff and Aikawa, 1998). This vector consists of two copies of a Lef/tcf responsive element upstream of a minimal thymidine kinase (TK) promoter which drives expression of a CAT gene. A linked RSV promoter driving expression of human growth hormone (hGH) was used for normalization. Assays to measure Wnt-1 dependent transcription from the Lef/tcf reporter construct were performed as described (Papkoff and Aikawa, 1998). The experiment shown in Fig. 5 was performed in triplicate and repeated twice. Monoclonal antibodies against the HA epitope, the Flag epitope and β-catenin were purchased from Boehringer, Kodak, and Transduction Labs, respectively.

Transfections, preparation of cell extracts, immunoprecipitations, western blot analysis

L cells were transfected with the indicated vectors using Lipofectamine (Gibco/BRL) as directed by the manufacturer. The amount of DNA for each transfection was kept at a fixed concentration and, CMV-eGFP was used as a 'filler' DNA where needed. Cell extracts were prepared between 24 and 48 hours for further analysis. For immunoprecipitations and affinity precipitations, extraction buffer consisted of 1% Triton-X-100, 10 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 1% aprotinin, 20 μg/ml leupeptin, 20 μg/ml PMSF, 2 ug/ml pepstatin, 100 mM sodium fluoride, 50 mM β-glycerophosphate and 1.0 mM sodium othrovanadate. Cell extracts were clarified by centrifugation in a refrigerated microfuge. Protein assays using a BCA kit (Pierce Chemical Co.) were performed on clarified cell extracts to ensure that protein equivalent aliquots were used for immunoprecipitations. Immunoprecipitations were performed as described (Papkoff, 1997). For affinity precipitation, clarified cell extracts were incubated with Sepharose beads (Pharmacia) bound to a GST-fusion protein consisting of the entire cytoplasmic domain of E-cadherin, as described (Papkoff, 1997). For all experiments washed immune complex pellets were boiled in Laemmli sample buffer and separated in a SDS-10% acrylamide gel followed by western immunoblot analysis with indicated antisera. Where indicated, cell extracts were analyzed without immunoprecipitation, for Wnt-1 or FrzA proteins.

Co-cultures and media transfer

For the experiment shown in Fig. 2, a first set of L cells were transfected with expression vectors encoding either eGFP (C) or HA-tagged Wnt-1. Next, conditioned media harvested from another set of cells transfected with vectors encoding either eGFP (C) or Flag-tagged FrzA was added to the first set of washed cells as indicated. Forty-eight hours after the transfection and media transfer, cells were rinsed with PBS followed by solubilization and immunoprecipitation with the appropriate antisera. For the experiment shown in Fig. 4, L cells were transfected with expression vectors for Wnt-1, FrzA or eGFP (control). Twenty-four hours later the transfected cells were resuspended and mixed together in the combinations indicated. Following a 24 hour co-culture, the cells were solubilized and analyzed by immunoprecipitation and western immunoblot.

RESULTS

Wnt-1 associates with FrzA upon co-expression in L cells

A potential association between FrzA and either Wnt-1 or Wnt-5a was first evaluated by co-transfection of these proteins into tissue culture cells. For this purpose, expression vectors, encoding either Flag-tagged FrzA or HA-tagged Wnt-1 (-5a), were constructed. The FrzA expression vector was co-transfected into L cells with either a Wnt-1 expression vector or with an eGFP expression vector as a control. Cell extracts were prepared and immunoprecipitated either with an

antiserum against the Flag-tag or with an antiserum against the HA-tag, followed by western immunoblot analysis with either anti-Flag or anti-HA antisera to visualize complex formation. Wnt-1 is readily detected in a FrzA immunoprecipitate (Fig. 1A, track 2) and conversely, FrzA is detected in a Wnt-1 immunoprecipitate (Fig. 1B, track 2). Four species of Wnt-1 protein, corresponding to the multiple glycosylated forms characterized previously (Papkoff, 1989; Papkoff et al., 1987), are recognized in the transfected cells and a single band corresponding to FrzA protein is detected.

Other studies have suggested that Wnt proteins can be divided into two classes based on functional assays; with Wnt-1 as the prototype of the first class and Wnt-5a of the second (Cadigan and Nusse, 1997; McMahon and Moon, 1989; Olson et al., 1991; Shimizu et al., 1997; Wong et al., 1994). Since FrzA associated with Wnt-1 we next tested whether FrzA was also capable of binding to Wnt-5a. A parallel analysis to that used to show FrzA binding to Wnt-1 (Fig. 1A,B) revealed that FrzA does not associate with Wnt-5a. No FrzA and Wnt-5a can be detected in association with each other upon co-transfection and immunoprecipitation, despite the presence of both of these proteins in the cell extract (Fig. 1C and D).

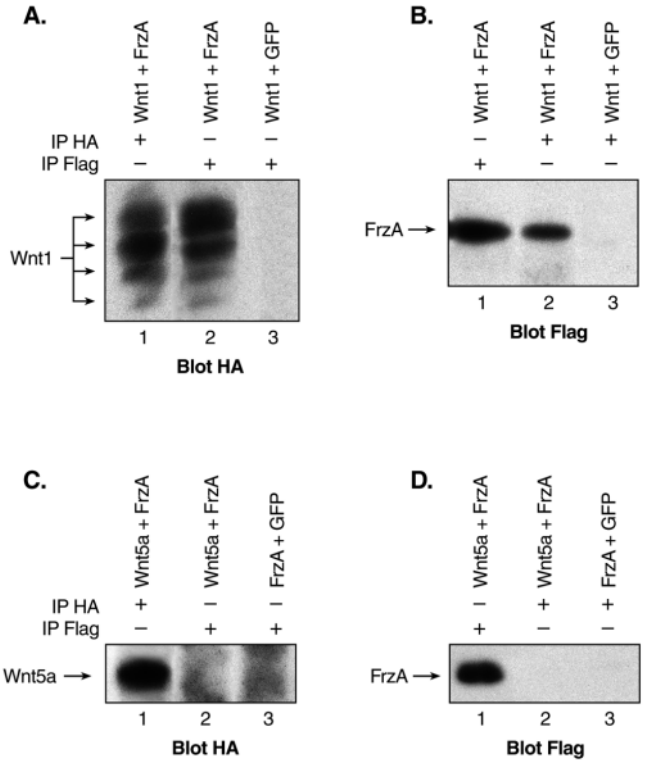

Fig. 1. FrzA co-immunoprecipitates with Wnt-1 but not Wnt-5a. (A,B) L cells were co-transfected with various combinations of expression vectors encoding HA-tagged Wnt-1, Flag-tagged FrzA or eGFP as a control. (C,D) L cells were co-transfected with various combinations of expression vectors encoding HA-tagged Wnt-5a, Flag-tagged FrzA or eGFP. Forty-eight hours after transfection, cells were extracted and immunoprecipitated with either an antiserum against HA (IP HA) or an antiserum against Flag (IP Flag) followed by western immunoblot with the same antibodies. All of the panels shown are from the same experiment.

Soluble FrzA binds to Wnt-1 transfected cells

Previous studies have shown that Wnt-1 is secreted from cells and remains associated with the cell surface, rather than diffusing into the culture medium (Papkoff and Schryver, 1990). In contrast to Wnt-1, FrzA protein is secreted and released into the tissue culture fluid (Xu et al., 1998), this paper. On the basis of these biochemical properties, we next asked whether soluble FrzA could bind specifically to the surface of Wnt-1-expressing cells. For this purpose, L cells were transfected with expression vectors encoding either HA-tagged Wnt-1 or a control (eGFP). The transfected cells were then cultured in the presence of media conditioned by cells expressing Flag-tagged FrzA or by control cells. Cells were rinsed and extracts were analyzed by western immunoblot with antisera against HA to detect the presence of Wnt-1 in the transfected cells or with antisera against Flag to detect the presence of FrzA bound to the transfectants. Results of these studies revealed that soluble FrzA can associate with Wnt-1 expressing cells (Fig. 2, track 4) but not with control cells (Fig. 2, track 2).

FrzA expression blocks a Wnt-1 induced increase in the free pool of β-catenin

Since FrzA is capable of binding to Wnt-1, it was of interest to determine if it was also able to modulate the biochemical effects of Wnt-1 signaling. To address this question we tested whether FrzA could block the ability of Wnt-1 to induce an increase in the free pool of β-catenin. Upon transfection of L cells with an expression vector for Wnt-1, there was a substantial induction of an uncomplexed pool of β-catenin (Papkoff, 1997, and Fig. 3). Expression of FrzA alone had no effect on β-catenin levels, however, when co-expressed with Wnt-1, FrzA blocked the Wnt-1 dependent increase in the free pool of β-catenin (Fig. 3). Based on these findings and on the FrzA binding data (Fig. 2) it was also of interest to determine whether FrzA was capable of affecting Wnt-1 signaling in neighboring cells. Separate dishes of L cells were transfected with expression vectors encoding Wnt-1, FrzA or a control gene (eGFP). Twenty-four hours later the transfected cells were removed from the dish and mixed together in various combinations as indicated. Following 24 hours of incubation,

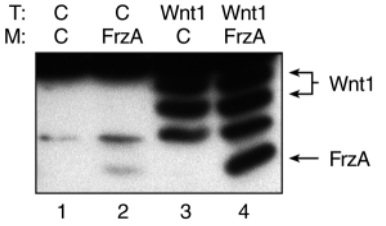

Fig. 2. Soluble FrzA binds to Wnt-1 transfected cells. L cells were transfected with expression vectors encoding either eGFP (C) or HA-tagged Wnt-1 (Wnt-1), then cultured in the presence of conditioned media harvested from cells transfected with expression vectors encoding either eGFP (C) or Flag-tagged FrzA (FrzA). Treated cells were then rinsed, solubilized and clarified cell extracts were analyzed by western blot with a combination of antisera against HA and Flag epitopes to detect both the expression of Wnt-1 in the cells and the FrzA bound to these cells. (T) indicates the specific vector transfected, (M) indicates the source of conditioned medium.

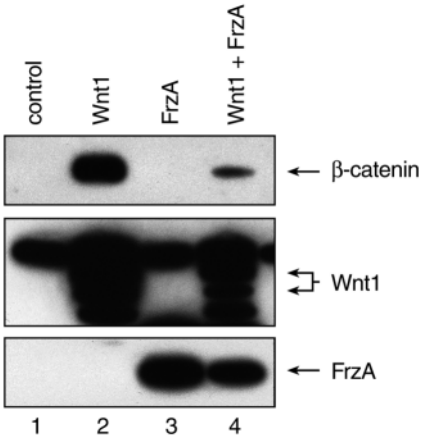

Fig. 3. Co-expression of FrzA abrogates a Wnt-1 induced increase in the free pool of β-catenin. L cells were transfected with an eGFP expression vector (control), with either Wnt-1 (Wnt-1) or FrzA (FrzA) expression vectors alone or with a combination of Wnt-1 and FrzA expression vectors (Wnt-1 + FrzA). Forty-eight hours after transfection, cell extracts were prepared and analyzed for levels of uncomplexed β-catenin by affinity precipitation followed by western immunoblot with an antiserum against β-catenin. Aliquots of the cell extracts were also analyzed by western immunoblot with antisera against HA- or Flag-epitopes for the detection of Wnt-1 and FrzA protein expression, respectively.

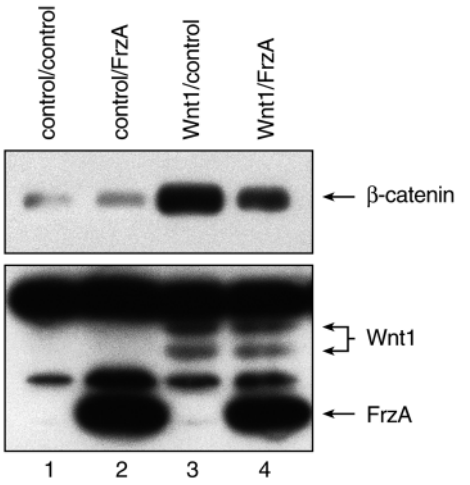

Fig. 4. Paracrine regulation of Wnt-1 signaling by FrzA. L cells were transfected with vectors encoding either eGFP (control), Wnt-1 (Wnt-1) or FrzA (FrzA). Twenty-four hours later the transfected cells were removed from the dish and mixed together in the indicated combinations. Following an additional 24 hours, cells were extracted and uncomplexed pools of β-catenin were analyzed by affinity precipitation followed by western immunoblot (upper panel). Aliquots of the cell extracts were also analyzed by western immunoblot with antisera against the HA- and Flag-epitope tags to verify the expression of Wnt-1 and FrzA, respectively (lower panel).

the co-cultures were harvested and analyzed for the β-catenin levels. When cells expressing Wnt-1 were mixed with cells expressing FrzA the ability of Wnt-1 to increase the free pool of β-catenin was attenuated, although not completely blocked (Fig. 4). Mixing of control cells, expressing eGFP, with Wnt-1 expressing cells did not diminish the ability of Wnt-1 to elevate the free pool of β-catenin (Fig. 4).

The increased free pool of β-catenin that occurrs as a consequence of Wnt-1 signal transduction forms a complex with transcription factors of the Lef/tcf family, leading to the activation of a Lef/Tcf dependent promoters (Clevers and Van De Wetering, 1997; Nusse, 1997). Therefore, we analyzed the effect of FrzA co-expression upon Wnt-1 activation of a Lef/Tcf responsive promoter. The reporter vector used for this analysis consisted of a Lef/Tcf response element placed upstream of a minimal TK promoter, driving expression of a CAT reporter gene. L cells were transfected with the reporter gene plus various combinations of genes encoding Wnt-1, FrzA and eGFP (control). Wnt-1 induced gene expression from a Lef/tcf-responsive promoter construct in a transient transfection assay (Fig. 5). Co-expression of FrzA with Wnt-1 substantially decreased the ability of Wnt-1 to modulate transcription whereas, FrzA alone had no effect on the the Lef/tcf-responsive reporter gene (Fig. 5).

DISCUSSION

Wnt-1 signal transduction is initiated upon binding of secreted Wnt-1 protein to a seven transmembrane receptor of the Frizzled family (Perrimon, 1996). The identification of another gene family encoding secreted proteins resembling the cysteine-rich, ligand binding domain of Frizzled receptors (sFRP) suggested the presence of an additional level of complexity in the regulation of Wnt signaling (Moon et al., 1997; Zorn, 1997). Here we demonstrate that Wnt-1 can physically associate with a specific sFRP family member,

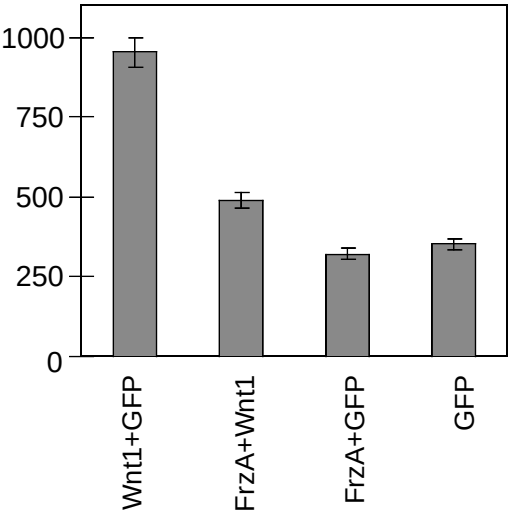

Fig. 5. FrzA blocks Wnt-1 stimulated transcription from a Lef/tcf-dependent reporter construct. A reporter construct that contains two copies of a Lef/tcf-responsive transcriptional element, placed upstream of a minimal TK promoter, driving expression of a CAT gene was co-transfected with expression vectors for eGFP (control), Wnt-1, FrzA, or Wnt-1 plus FrzA. Thirty-six hours after transfection cells were harvested and analyzed for expression of the CAT reporter gene as well as expression from a linked hGH gene for normalization of transfection.

FrzA, and that this association leads to an attenuation of the signal transduction responses normally activated by Wnt-1.

There exists a multiplicity of Frizzled receptors, at least 18 murine Wnt proteins and a growing family of sFRPs (Cadigan and Nusse, 1997; Perrimon, 1996; Zorn, 1997). Whereas some Frizzled/Wnt binding pairs have been established based upon physical association and biological activity upon co-expression, a complete profile of the binding specificities between the various ligand-receptor pairs remains to be defined. The data presented here establish that FrzA protein specifically associates with Wnt-1 but does not associate with Wnt-5a. In other experiments we were unable to demonstrate significant binding of a different sFRP, Fritz (Frzb-1), to either Wnt-1 or Wnt-5a (data not shown). These data confirm the notion that there is specificity in the pairing of different Wnt proteins with Frizzled receptors and sFRPs and identifies Wnt-1 as a specific partner for FrzA. The apparent difference in sFRP binding capability of Wnt-1 and Wnt-5a is consistent with previous studies that documented different biological activities for Wnt-1 versus Wnt-5a, despite the fact that the biochemical properties of these two Wnt proteins are quite similar (Smolich et al., 1993). Furthermore, these findings are consistent with the recent observation that FrzA expression can abrogate the axis duplicating activity of Wnt-1 in *Xenopus* but has no effect on the ability of Wnt-5a to suppress morphogenetic movements (Xu et al., 1998). Taken together the available data suggest that the binding specificity between particular Wnt family proteins and Frizzled receptors leads to diverse signaling effects. The apparent specificity of binding between sFRPs and Wnt proteins adds further complexity and fine tuning to this regulatory network.

Data presented here demonstrate that Wnt-1 and FrzA can associate with each other upon co-expression within the same cell. Wnt-1 protein processing and secretion has been extensively characterized and a 36 kDa unglycosylated form as well as sequentially glycosylated species of 38 kDa, 40 kDa and 42 kDa have been identified in cell extracts (Papkoff, 1989; Papkoff et al., 1987; Papkoff and Schryver, 1990). The more mature forms, 40 kDa and 42 kDa, are the predominant species identified in cells that efficiently process and secrete Wnt-1 and these forms are the major species represented at the cell surface or associated with extracellular matrix (Papkoff and Schryver, 1990). In the experiments presented here, there is an increased representation of the 40 kDa and 42 kDa Wnt-1 protein species, relative to the 36 kDa and 38 kDa, in association with FrzA compared to the relative amounts of these proteins in total cell extracts. In addition to documenting an association between Wnt-1 and FrzA within the same cell, we also showed that soluble FrzA, when provided in conditioned medium, can bind to Wnt-1 expressing cells. Furthermore, when soluble FrzA is added to Wnt-1 expressing cells, where the secreted Wnt-1 protein remains associated with the cell surface, only the 40 kDa and 42 kDa Wnt-1 proteins are co-immunoprecipitated. These findings imply that FrzA, preferentially associates with the more mature, cell surface forms of Wnt-1. We previously showed that essentially all of the secreted Wnt-1 protein remained associated with the cell surface (Papkoff and Schryver, 1990). In the current study, examination of media from cells co-expressing both Wnt-1 and FrzA did not reveal any Wnt-1 protein (S. Dennis and J. Papkoff, unpublished results). This finding suggests that when FrzA associates with Wnt-1, the complex also remains cell associated and that FrzA cannot escort Wnt-1 into the medium.

Co-expression of FrzA with Wnt-1 blocked a Wnt-1 dependent increase in uncomplexed β-catenin levels as well as increased transcription from a Lef/tcf-dependent reporter gene construct. These findings suggest that, when expressed by the same cells FrzA could modulate Wnt protein function in an autocrine fashion. A paracrine interaction was demonstrated in other experiments where co-culture of FrzA producing cells with Wnt-1 expressing cells led to an attenuation of the Wnt-1 dependent increase in free β-catenin levels. Together the findings presented here suggest that FrzA binding to Wnt-1 can modulate its signaling effects in both an autocrine and paracrine fashion.

The data presented here provide evidence that sFRP proteins act as negative regulators of Wnt protein signaling by binding and sequestration of the Wnt proteins from their Frizzled receptors. In support of this model, data obtained in *Xenopus* studies suggest that FrzA and other sFRP proteins can, in fact, antagonize phenotypes induced by ectopic expression of Wnt proteins (Leyns et al., 1997; Lin et al., 1997; Mayr et al., 1997; Wang et al., 1997a,b; Xu et al., 1998). It is conceivable that sFRP proteins could either inhibit or facilitate the diffusion of Wnt proteins, which generally remain closely associated with producing cells. Modulation of the extracellular distribution of Wnt proteins could have profound consequences in a developmental setting, particularly with respect to pattern formation. Since FrzA is highly expressed by endothelial cells (Xu et al., 1998) this suggests that Wnt signaling may play an important role in normal vascular biology or angiogenesis. It will be important to define the specific pairing relationships between all of the various Wnt, Frizzled and sFRP proteins and to elucidate the consequent effects on Wnt protein signaling in diverse settings.

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