The establishment of the vertebrate body plan requires inductive signals from the dorsal blastopore lip, also known as Spemann's organizer, in amphibians (Spemann and Mangold, 2001; De Robertis and Aréchaga, 2001; Stern, 2001). Surprisingly, many of the secreted factors expressed specifically in the organizer were found to be antagonists of growth factor signaling, such as the BMP (Bone Morphogenetic Protein) antagonists Chordin, Noggin, Follistatin, Xnr3 and Cerberus (reviewed by De Robertis et al., 2000). Studies in vertebrates as well as invertebrates indicated that a BMP signaling gradient, which is established in the early embryo by the localized secretion of these organizer specific inhibitors, plays a central role in the determination of the dorsoventral body axis.

Chordin is a large secreted protein abundantly expressed in Spemann's organizer, reaching extracellular concentrations in the 6-12 nanomolar range (Piccolo et al., 1996). Chordin binds directly to BMP and prevents BMP binding to its cognate receptor, causing dorsalization of the embryo in overexpression studies (Sasai et al., 1995; Piccolo et al., 1996). The binding of Chordin to BMP is mediated by four cysteine-rich domains (CRs) of about 70 amino acids each (Larraín et al., 2000). A large number of additional extracellular proteins containing CR domains have now been identified, and interactions with BMP or TGFβ have been documented for many of them (reviewed in García-Abreu et al., 2002).

In Drosophila, the chordin homolog short gastrulation (sog) is expressed in ventral neuroectoderm and is required for the formation of neural tissue (François et al., 1994). In addition, Sog is necessary for the formation of the dorsal-most tissue of the fly embryo, the amnioserosa, which requires maximal BMP activity (Ross et al., 2001; Eldar et al., 2002).

In zebrafish, the ventralized chordino phenotype is caused by a loss-of-function mutation in the zebrafish chordin gene (Schulte-Merker et al., 1997; Hammerschmidt and Mullins, 2002). The neural plate and dorsal mesoderm are reduced, and ventral mesoderm is expanded in chordino mutants (Hammerschmidt et al., 1996a; Gonzalez et al., 2000). The opposite phenotype, dorsalization, is observed in bmp2b/swirl, bmp7/snaillhouse, Smad5/somitabun and Tolloid/mini-fin mutants (Mullins et al., 1996; Hammerschmidt and Mullins, 2002). In addition, chordino/swirl double mutants display the swirl phenotype, confirming that Chordin functions genetically as an anti-BMP (Hammerschmidt et al., 1996b). Recent studies on swirl/chordin genetic interactions indicate that in zebrafish, Chordin also functions in the formation of ventral...
tail fin tissue that requires maximal BMP signaling (Wagner and Mullins, 2002). This suggests that in vertebrates Chordin, like Sog in the amnioserosa of the fly, may promote BMP signaling as well.

In mouse, the targeted inactivation of *chordin* results in a ventralized gastrulation phenotype only in a low percentage of the homozygous mutant embryos. Most mutants lack anterior notochord and pharyngeal endoderm, causing a phenotype similar to human DiGeorge syndrome, but do not have gastrulation phenotypes (Bachiller et al., 2003). However, mice homozygous for mutations in *chordin* and *noggin* lack the forebrain, indicating that Noggin can partially compensate for the loss of Chordin (Bachiller et al., 2000).

In *Xenopus*, inhibition of Chordin production by morpholino oligonucleotides causes a phenotype similar to that of zebrafish *chordin*, with embryos developing with smaller heads and enlarged ventroposterior structures (Oelgeschläger et al., 2003). This relatively weak ventralization contrasts with the strong requirement for Chordin observed when the embryos are experimentally manipulated. Indeed, dorsalization of embryos by LiCl (Kao and Elinson, 1988), dorsal mesoderm induction by Activin (Green et al., 1992) and CNS induction by dorsal lip grafts, all show a complete dependence on the presence of Chordin (Oelgeschläger et al., 2003).

Tsg is a co-factor of Chordin that can bind both to BMP and to Chordin, generating trimeric complexes that antagonize BMP signaling (Oelgeschläger et al., 2000; Chang et al., 2001; Scott et al., 2001; Larraín et al., 2001). The cleavage of Chordin by the Xolloid (Xld)/Tolloid (Tld) zinc-metalloprotease generates protein fragments that include intact BMP-binding cysteine-rich modules (CRs) and retain anti-BMP activity (Larrain et al., 2000). Tsg facilitates the cleavage of Chordin by Tolloid (Scott et al., 2001; Larraín et al., 2001) and antagonizes the residual anti-BMP activity of the proteolytic cleavage products of Chordin (Oelgeschläger et al., 2000; Larraín et al., 2001). Thus, in this second aspect of its activity Tsg behaves as a pro-BMP. The cleavage of Chordin by Xld/Tld constitutes the molecular switch that controls the anti-BMP and pro-BMP activities of Tsg protein in this biochemical pathway (Larrain et al., 2001).

The multifunctional properties of Tsg hamper the analysis of its function in embryonic patterning. For example, in *Xenopus* embryos overexpression of xTsg has a pro-BMP effect, resulting in ventralization (Oelgeschläger et al., 2000). In zebrafish, overexpression of zTsg causes an anti-BMP, dorsalized phenotype (Ross et al., 2001). The difference in phenotypes may be attributed to a lower endogenous activity of Tolloid during early zebrafish development (Connors et al., 1999; Larraín et al., 2001). Indeed, the ventralizing activity of xTsg in *Xenopus* could be reversed by overexpression of a dominant-negative Xolloid (Larrain et al., 2001).

In the present study, we dissected the anti-BMP and pro-BMP activities of xTsg by generating a series of point mutations that affected specifically one or the other activity of xTsg. The Tsg protein contains two evolutionary conserved cysteine-rich domains. The C-terminal cysteine-rich region does not have any significant homology to known protein motifs and its role in the biochemical activities of Tsg is unknown. The N-terminal cysteine-rich domain of Tsg has some homology to the BMP-binding modules of Chordin (CRs) and is necessary and sufficient for the direct interaction of xTsg with BMP (Oelgeschläger et al., 2000). As shown here, mutations in the N-terminal domain generated mutant xTsg proteins that no longer bound BMP and had greatly enhanced ventralizing activities, preventing the formation of CNS and dorsal mesoderm in *Xenopus* embryos. This hyperventralizing activity of xTsg mutants required an intact C-terminal domain, and phenotypes were much stronger than those caused by Chordin loss-of-function, both in *Xenopus* and zebrafish embryos. Hyperventralizing xTsg mutations specifically antagonized proteins containing BMP-binding modules of the Chordin type. Our data indicate that xTsg inactivates CR-modules through its C-terminal conserved domain and that xTsg may interact, in addition to Chordin, with other CR-containing proteins required for the regulation of early dorsoventral patterning.

**MATERIALS AND METHODS**

**DNA constructs and morpholinos** Site-directed mutagenesis was performed with the QuickChange mutagenesis kit (Stratagene). Point mutations were introduced into *Xenopus* and mouse Tsg constructs lacking the endogenous signal peptide and fused to the signal peptide of *Xenopus* Chordin followed by a FLAG-tag (pCS2-ChdN-Tsg) (Oelgeschläger et al., 2000; Larraín et al., 2001). For the mutations in S36, S54, C59, C180 and C198 in xTsg, and C185 and C203 in mouse Tsg, the respective amino acids were mutated to Alanine; W67 in *Xenopus* Tsg and the corresponding W66 in mouse Tsg were mutated into Glycine. A combination of two anti-*chordin* morpholinos was used as described (Oelgeschläger et al., 2003).

**Embryo manipulations and *chordino* genotyping** Microinjections, in situ hybridization and RNA synthesis were performed as described (Piccolo et al., 1997; Oelgeschläger et al., 2000; Sive et al., 2000). For LiCl rescue experiments, embryos were microinjected at the two to four cell stage four times with a total of 2 ng TsgW67G or 8 ng of a 1:1 mixture of the two Chordin morpholinos. Embryos at the 32-64 cell stage were treated for 29 minutes with 120 mM LiCl in 0.1xMBS saline (Larrain et al., 2000; Sive et al., 2000). The dorsoanterior index (DAI) was determined at stage 30 (Kao and Elinson, 1988). Treatment of animal cap explants with human recombinant Activin protein (R&D Systems) was as described (Piccolo et al., 1999). RNA was isolated at stage 25. RT-PCR conditions and PCR primers used have been described elsewhere (Sasai et al., 1995) (http://www.hhmi.ucla.edu/derobertis/index.html). To genotype *chordin* mutant embryos two primers were used for PCR: chd-2 GCA GAA ACG TCT ACG TT TCC and chd-3 CGT TTT AGT TGG TGC TCT TGA CG. Following digestion with *MspI*, the wild-type allele was digested whereas the mutant allele was not.

**Protein biochemistry** *Xenopus* Tsg and the mutant Tsg proteins were affinity purified using the anti-FLAG M2 affinity gel column (Sigma). The anti-FLAG column (0.5 ml) was washed once with 5 ml 0.1 M glycine, pH 3.5 and three times with 5 ml aliquots of TBS (50 mM Tris HCl, 150 mM NaCl, pH 7.4). Conditioned medium from human 293 cells transfected with pCS2-ChdN-xTsg or Tsg point mutants was harvested and loaded directly onto the column. After washing three times with 10 ml TBS, the bound protein was eluted with five aliquots of 1 ml TBS containing 100 μg/ml FLAG peptide (Sigma). Protein concentrations were estimated by comparison with BSA standards after Coomassie Blue staining. Analyses of protein secreted by animal cap explants were performed as described (Oelgeschläger et al., 2000). The supernatant was used for western blots probed with an
antibody specific for the inter-repeat region of Chordin (α-I-Chd) (Piccolo et al., 1997) that had been blot-affinity purified (Larraín et al., 2001). For the expression of proteins in co-cultures, 293T cells were independently transfected with mouse Chordin, Bmp4, Tsg and TsgW66A expression plasmids using FuGENE (Roche), and chemical crosslinking and immunoprecipitation experiments carried out as described (Oelgeschläger et al., 2000; Larraín et al., 2001).

RESULTS

Tsg mutations reveal distinct activities for the N and C terminus

The N-terminal cysteine-rich domain of xTsg shares homology with the CRs of Chordin (Fig. 1A) (Oelgeschläger et al., 2000). Chordin CRs mediate the direct binding of Chordin to BMPs and are characterized by 10 cysteines spaced in a characteristic manner (Larraín et al., 2000). The conserved cysteines, as well as a conserved tryptophan, and potential glycosylation sites, were mutated (Fig. 1A,B) and tested by microinjection into Xenopus embryos.

Embryos overexpressing wild-type xTsg mRNA developed with reduced head structures, distended trunk endoderm and a posteriorized anus that detached from the main endodermal mass later in development (Fig. 1D). Three of the point mutants examined caused a hyperventralized phenotype when compared with xTsg. Embryos microinjected with mRNA coding for xTsgS36A, xTsgC59A and xTsgW67G lost head and dorsal structures and had an enlargement of ventral blood islands (Fig. 1E,G,H and data not shown). These phenotypes were indistinguishable from those of Xenopus embryos overexpressing mRNAs encoding BMP4 or BMP7 (not shown).

The C-terminal cysteine-rich domain of Tsg did not show any significant homology to known protein motifs in the databases but is conserved across Tsgs from different species. Mutations in several of its conserved cysteine residues were tested, two of which (xTsgC180A, xTsgC198A) had a phenotypic effect causing dorsalization, with reduced trunk and enlarged head structures (Fig. 1I and data not shown).

When W67G, the strongest ventralizing (pro-BMP) mutation, was combined with one of the dorsalizing mutations (C198A), the resulting double mutant (xTsgW67G+C198A) had almost no ventralizing activity in overexpression experiments (Fig. 1J, compare with 1H). However, in a subset of embryos injected with this double mutant weak phenotypes, including reduced head structures and defects in tail development could be observed. Mutation of a potential glycosylation site (xTsgS54A) resulted in a typical xTsg phenotype, with reduced head structures and a posteriorized anus, although the phenotype was stronger than its wild-type counterpart (compare Fig. 1D,F). S54 seems to be a site of glycosylation in vivo, as the mutant protein had faster electrophoretic mobility (Fig. 1K, compare lanes 1 and 3). Perhaps the enhanced activity of this glycosylation site mutant is due to increased diffusibility in the embryo.

We conclude from these data that mutations in the N-terminal cysteine-rich domain enhanced the ventralizing (pro-BMP) activity of Tsg, whereas those in the C-terminal domain caused the opposite effect, dorsalization. The phenotype of the double mutant further demonstrated that the hyperventralizing activity of xTsg mutants requires the function of the C-terminal cysteine-rich domain.

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**Fig. 1.** The ventralizing activity of Tsg resides in its C-terminal cysteine-rich domain. (A) The Tsg mutations presented here. (B) Alignment of the N-terminal cysteine rich domain of Tsg proteins from Xenopus (xTsg), mouse (mTsg), human (hTsg), Drosophila (dTsg, dTsg2) and Chordin CR1. (C-J) Stage 42 wild-type control embryo (C) and embryos radially microinjected at the four-cell stage with a total of 2 ng xTsg mRNA (D), xTsgS36A (E), xTsgS54A (F), xTsgC59A (G), xTsgW67G (H), xTsgC198A (I), xTsgW67G+C198A (J). Each construct was analyzed in at least three independent experiments with n>30. (K) xTsg and mutant xTsg proteins secreted by dissociated animal cap cells after microinjection of 2 ng of the indicated mRNAs.
The hyperventralizing xTsg\textsuperscript{W67G} mutant

To compare the ventralizing effects of wild-type xTsg and Tsg\textsuperscript{W67G} on CNS patterning, we analyzed the neural plate marker \textit{Sox}2 by in situ hybridization. At early neurula stages, microinjection of 1 ng of xTsg mRNA led to reduction of the neural plate and the formation of a ring of \textit{Sox}2 positive cells surrounding the slit-shaped blastopore (Fig. 2A,B). This ring may indicate a posteriorization of the embryo, and correlated with the posteriorized anus phenotype at tadpole stages. The mass of posteriorized and detached endoderm expressed \textit{Sox}2, which marks the proctodeum (Fig. 2K,L) (Chalmers et al., 2000). At higher concentrations (4 ng), xTsg mRNA reduced the neural plate further, indicating that the wild-type protein has pro-BMP effects (Fig. 2C) (Oelgeschläger et al., 2000). Results were different when the hyperventralizing mutant Tsg\textsuperscript{W67G} was microinjected: the reduction of the neural plate was much more severe and the ring of \textit{Sox}2-positive cells as well as the posteriorized anus were not seen (Fig. 2D,I).

Staining for \textit{N-tubulin}, a marker for differentiated neurons, confirmed the decrease in anterior neural plate caused by the microinjection of xTsg mRNA. In these embryos, the lateral sensory (Rohon-Beard) neurons formed a ring surrounding the blastopore slit (Fig. 2E,F). Microinjection of xTsg\textsuperscript{W67G} led to an almost complete inhibition of early neurogenesis (Fig. 2H) similar to, although more severe than, microinjection of \textit{Xolloid} mRNA (Fig. 2G). The anti-neural effect of Tsg\textsuperscript{W67G} was mediated by increased BMP signaling, as co-injection of mRNA encoding dominant-negative BMP receptor (\textit{tBR}) rescued the formation of dorsoanterior CNS structures such as forebrain and eyes (Fig. 2J). Most of the rescued embryos had a posteriorized anus that detached from the trunk (Fig. 2J). This phenotype was also observed in embryos injected into the animal pole with 1 ng of constitutively active BMP receptor or Smad5 mRNA (data not shown) (Beck et al., 2001). Thus, at this point it is not possible to establish whether the detachment of the proctodeum represents a pro-BMP or anti-BMP activity.

In histological sections, embryos overexpressing wild-type xTsg showed a mild reduction of the spinal cord and somites (Fig. 2M,N) and in some cases a hypoplastic notochord (data not shown) (Oelgeschläger et al., 2003). By contrast, embryos injected with Tsg\textsuperscript{W67G} showed considerable reduction of the brain, spinal cord, notochord and somites (Fig. 2O) (data not shown).

We conclude that xTsg mRNA inhibited development of the anterior neural plate and posteriorized the embryo. In the hyperventralizing mutant Tsg\textsuperscript{W67G}, the pro-BMP activities of wild-type xTsg were exacerbated.

Tsg and Tsg\textsuperscript{W67G} antagonize dorsalization of mesoderm by Activin

In *Xenopus*, formation of dorsal mesoderm is mediated by a horizontal signal secreted by Spemann’s organizer (Dale and Slack, 1987). This signal can be mimicked by treatment of ectodermal explants with Activin protein (Green et al., 1992; Dyson and Gurdon, 1998). Activin induces the expression of \textit{chordin} in ectodermal explants, and we have recently shown that this expression of endogenous Chordin is required for dorsal mesoderm induction (Oelgeschläger et al., 2003). Treatment of ectodermal explants with 2 ng/ml Activin triggered the convergence-extension movements that accompany dorsal mesoderm formation (Fig. 3B). In explants microinjected with xTsg or Tsg\textsuperscript{W67G}, animal cap elongation was blocked (Fig. 3C,D). RT-PCR analysis confirmed that the induction by Activin of the...
dorsal mesodermal markers MyoD and α-Actin and of the pan-neural marker NCAM was inhibited by wild-type xTsg or Tsg\textsubscript{W67G} mRNAs (Fig. 3F). Overexpressed xTsg is known to induce degradation of Chordin protein secreted by Spemann’s organizer (Larrain et al., 2001). As expected, microinjection of xTsg mRNA led to a decrease of full-length Chordin protein secreted by Activin-treated animal caps (Fig. 3E, lanes 2, 3). By contrast, Tsg\textsubscript{W67G} increased the amount of Chordin protein secreted by Activin-treated animal caps (Fig. 3E, lanes 2, 3). By contrast, Tsg\textsubscript{W67G} increased the amount of Chordin protein secreted by Activin-treated animal caps (Fig. 3E, lanes 2, 3). Thus, although full-length Chordin protein was abundantly produced in animal caps in the presence of Tsg\textsubscript{W67G} (Fig. 3E, lane 4), this Chordin protein was inactive in mesodermal dorsalization (Fig. 3F, lane 5).

We conclude that xTsg and Tsg\textsubscript{W67G} antagonize Activin dorsalization by different molecular mechanisms. The ventralizing activity of wild-type xTsg may be explained by the increased degradation of Chordin, but Tsg\textsubscript{W67G} caused the accumulation of full-length Chordin protein that had lost its dorsalizing (anti-BMP) activity.

**Hyperventralizing Tsg proteins do not bind BMP**

To test the activity of Tsg proteins, Tsg\textsubscript{C59A} and Tsg\textsubscript{W67G} inactivates CR modules

To test whether binding to Chordin was affected by the hyperventralizing mutations xTsg and Tsg\textsubscript{W67G}, we carried out a series of functional and biochemical experiments. As reported previously, xTsg RNA induced expression of the cement gland marker XAG-1 in animal cap explants (Fig. 4A, lane 3) (Chang et al., 2001; Larrain et al., 2001). However, this anti-BMP effect was not sufficient to induce the expression of the pan-neural marker NCAM, which requires even lower levels of BMP signaling (Fig. 4A). We tested the various xTsg point mutants in animal cap explants and found that, like wild-type xTsg, the hyperventralizing mutant xTsg\textsubscript{C198A} induces XAG-1 but not NCAM expression (Fig. 4A, lane 5). The hyperventralizing mutations xTsg\textsubscript{S36A}, xTsg\textsubscript{C59A} and Tsg\textsubscript{W67G} did not induce XAG-1 expression in animal cap explants (Fig. 4A, lane 4 and data not shown). One possible explanation for this difference was that the N-terminal mutations might prevent binding of xTsg to BMP.

To test this biochemically, we prepared affinity-purified xTsg proteins and incubated them with recombinant BMP4 protein. BMP4/xTsg complexes were immunoprecipitated via the N-terminal FLAG-tag of the Tsg proteins, and immunoprecipitated proteins were visualized with antibodies specific for BMP4. Wild-type xTsg bound BMP4 protein (Fig. 4B, lane 4) (Oegenschläger et al., 2000). By contrast, two N-terminal mutations that were unable to induce XAG-1 expression in animal caps, Tsg\textsubscript{C59A} and Tsg\textsubscript{W67G}, did not bind BMP4 at detectable levels (Fig. 4B, lanes 2 and 3). Thus, the induction of XAG-1 in animal caps by overexpressed xTsg correlated with a mild anti-BMP activity of xTsg mediated by direct binding to BMP. Importantly, Tsg\textsubscript{C59A} and Tsg\textsubscript{W67G}, which were devoid of BMP-binding activity, had strong ventralizing (pro-BMP) activity in biological assays.

**Tsg\textsuperscript{W67G} does not form ternary complexes with Chordin and BMP**

To test whether binding to Chordin was affected by the mutations, affinity-purified mutant xTsg proteins were incubated with full-length *Xenopus* recombinant Chordin protein, and complexes chemically crosslinked with disuccinimidyl suberate (DSS). Wild-type xTsg protein formed a high molecular weight complex in the presence of Chordin, corresponding to a dimer of xTsg bound to Chordin (Fig. 4C, lane 2). A similar complex was observed in the presence of Tsg\textsubscript{W67G} and Chordin protein (Fig. 4C, lane 4). By contrast, Chordin-Tsg complexes were not detectable using the C-terminal mutant Tsg\textsubscript{C198A} (Fig. 4C, lane 6).

We conclude from these biochemical experiments that the hyperventralizing mutant Tsg\textsubscript{W67G} is not affected in its ability to bind to Chordin, and that dorsalizing mutations in the C-terminal domain prevent binding of xTsg to Chordin.

**Fig. 3.** Wild-type xTsg and the hyperventralizing mutant xTsg\textsubscript{W67G} antagonize the dorsalization of mesoderm by Activin. (A–D) Animal cap explants were isolated at stage 8 and treated with 2 ng/ml Activin protein. Activin induces elongation (B). Microinjection of a total of 2 ng xTsg (C) or xTsg\textsubscript{W67G} (D) mRNA at the eight-cell stage into the animal blastomeres prevented elongation. (E) The accumulation of Activin-induced endogenous Chordin protein (lane 2) was reduced by wild-type xTsg mRNA injection (lane 3) and increased by xTsg\textsubscript{W67G} (lane 4). (F) RT-PCR analysis of animal cap explants treated with Activin after microinjection of 2 ng xTsg or xTsg\textsubscript{W67G} mRNA; both mRNAs inhibited the induction of dorsal mesodermal (MyoD, alpha-Actin) or neural (NCAM) marker genes by Activin. EF1α served as a loading control.
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Tsg protein with Chordin and BMP4 generated a ternary complex corresponding to a Tsg dimer bound to a BMP4 dimer and Chordin (Fig. 4D, lane 3).

By contrast, when Tsg W67G protein was used, only the Chordin-BMP4 complex was detectable (Fig. 4D, lane 4). When the same blot was incubated with a Flag-Tsg specific antibody, complexes of Chordin and TsgW67G were detectable (Fig. 4E, lane 4). As ternary complexes were not formed, this indicates that the full-length Chordin protein bound to TsgW67G lost its BMP4 binding ability. This result was surprising, as Chordin is a large protein containing four BMP-binding domains of the CR type. Tsg is a rather small molecule, five times smaller than Chordin, with only one BMP interaction domain. These results raise the possibility that TsgW67G might inactivate the BMP-binding activity of Chordin in an active way (see below).

The inability of TsgW67G to form ternary complexes with Chordin and BMP was confirmed using a co-culture system of human 293T kidney cells. Cell cultures separately transfected with expression constructs for mouse Chordin, BMP4, mTsg or mTsg W66G were mixed, cultured together for 24 hours, and proteins secreted into the culture medium were analyzed after chemical crosslinking. Even under these conditions, which greatly facilitate the detection of trimolecular complexes, the TsgW66G mutation precluded the binding of BMP4 to Chordin complexes (Fig. 4F, lane 6).

We conclude from these biochemical experiments that the hyperventralizing mutations prevent binding of Tsg to BMP4 but allow binding to full-length Chordin. Binding of xTsg W67G to Chordin prevents binding of BMP to either protein, and this inhibition correlates with the enhanced ventralizing activity of this Tsg mutant.

TsgW67G inhibits CR-modules
When Chordin is digested by the Tolloid/Xolloid
metalloprotease the molecule is cleaved twice, releasing intact CR modules that can still bind and inhibit BMP. Unlike full-length Chordin, individual CR modules do not form stable complexes with xTsg. However, wild-type xTsg has the ability to antagonize the residual anti-BMP activity of the proteolytic fragments (Oelgeschläger et al., 2000; Larraín et al., 2001). We tested the effects of Tsg\textsuperscript{W67G} on Xenopus CR1, a protein that mimics the N-terminal cleavage product. Microinjection of mRNA encoding xCR1 induced the expression of the anterior neural marker genes Rx\textsubscript{2a} and Six\textsubscript{3}, the pan-neural marker gene NCAM, and the cement gland marker XAG\textsubscript{1} in animal cap explants (Fig. 5A, lane 3). Co-injection of wild-type xTsg mRNA inhibited the expression of anterior neural marker genes but expression of XAG\textsubscript{1} was still observed (Fig. 5A, lane 4). Co-injection of Tsg\textsuperscript{W67G} mRNA completely blocked the induction of XAG\textsubscript{1} and of neural markers by xCR1 (Fig. 5A, lane 5). In addition, expression of the epidermal BMP target gene M\textsubscript{x2}, which was inhibited by xCR1, was restored by Tsg\textsuperscript{W67G} mRNA (Fig. 5A).

Thus, Tsg\textsuperscript{W67G}, like wild-type Tsg, can inactivate the anti-BMP activity of a CR-module. This was surprising, as we had previously assumed from biochemical experiments that xTsg inhibited CRs through mutual competition for BMP binding (Larraín et al., 2001). Because the hyperventralizing Tsg\textsuperscript{W67G} mutant does not bind BMP4, its effects on CR1 activity must be through a different, non-competitive, molecular mechanism.

**Tsg\textsuperscript{W67G} is specific for CR-containing proteins**

We next tested the effect of Tsg\textsuperscript{W67G} on other inhibitors of BMP signaling that lack CR-modules. Ventral injection of tBR mRNA resulted in the formation of partial secondary axes that were not affected by co-injection of Tsg\textsuperscript{W67G} mRNA (Fig. 5D,E). This shows that Tsg\textsuperscript{W67G} acts on the BMP signaling pathway, upstream of the BMP receptor. Microinjection of noggin mRNA induced secondary axes that were not affected by co-injection of Tsg\textsuperscript{W67G} mRNA (Fig. 5F,G). By contrast, axes induced by injection of xCR1 mRNA were abolished by co-injection of Tsg\textsuperscript{W67G} (Fig. 5B,C). As Tsg\textsuperscript{W67G} does not affect doryalization by tBR or Noggin, we conclude that the effects of Tsg\textsuperscript{W67G} in the embryo are specific for CR modules.

**Hyperventralizing Tsgs compared with Chordin loss of function**

Chordin downregulation after microinjection of morpholino oligonucleotides results in a moderate ventralization of Xenopus embryos comparable with that of chordin mutant zebrafish (Oelgeschläger et al., 2003). The phenotype of the hyperventralizing mutants xTsg\textsuperscript{W67G} and xTsg\textsuperscript{C59H} is much more severe, causing extensive loss of the CNS, particularly in the anterior (Fig. 1G,H). Striking pro-BMP effects of xTsg\textsuperscript{W67G} were observed in LiCl-treated Xenopus embryos. Embryos treated at the 32-cell stage with 120 nM LiCl expressed organizer genes such as Chordin throughout the marginal zone at gastrula and cause the development of embryos with radial head structures lacking trunk-tail structures (Fig. 6A,B; Dorsoanterior index, DAI=9.25, n=16). The effect of LiCl can be blocked by microinjection of Chordin morpholinos (Oelgeschläger et al., 2003). Even in the presence of LiCl, Chordin morpholinos produced a chordino-like weakly ventralized phenotype (Fig. 6D). Overexpression of Tsg\textsuperscript{W67G} had a strong effect on LiCl treated embryos. Microinjection of Tsg\textsuperscript{W67G} mRNA into each of the four blastomeres resulted in embryos that were strongly ventralized and lacked CNS and axial structures, despite having been treated with LiCl (Fig. 6C, DAI=1.1, n=11). Thus, the ventralizing effect of Tsg\textsuperscript{W67G} is much stronger than that of Chordin loss of function, and suppressed most dorsal development even in what should have been doryalized LiCl-treated embryos.

In zebrafish, the complete loss-of-function phenotype of Chordin is well characterized (Hammerschmidt, 1996a; Wagner and Mullins, 2002). As xTsg binds to Chordin and can promote Chordin degradation in overexpression studies (Oelgeschläger et al., 2000; Scott et al., 2001; Larraín et al., 2001), it was important to determine whether the effects of xTsg hyperventralizing mutants should be explained solely by antagonism of Chordin. We overexpressed wild-type xTsg and two xTsg hyperventralizing variants in zebrafish embryos. Wild-type xTsg mRNA dorsalized these embryos (Fig. 7A-D), as reported previously for zebrfish Tsg (Ross et al., 2001). The opposite effect, ventralization, is observed in Xenopus (Fig. 2B,C) (Oelgeschläger et al., 2000). This difference has been proposed to be due to lower levels of Tolloid activity during early zebrafish development (Connors et al.,

![Image](image-url)
As the hyperventralizing Tsg activities are specific for proteins containing CR modules (Fig. 5), these data indicate that other CR-containing proteins, in addition to Chordin, must be required for the establishment of dorsal cell fate in *Xenopus* and zebrafish development.

**DISCUSSION**

**The multiple activities of Tsg**

In an effort to separate the multiple activities of Tsg, we introduced point mutations in xTsg, concentrating on the evolutionarily conserved cysteine-rich domains located at the N and C termini. Three mutations in the N terminus at positions 36, 59 and 67 generated a series of mutants with increasing ventralizing (pro-BMP) activities. The strongest one, xTsgW67G, resulted in phenotypes corresponding to ventral belly pieces (‘Bauchstücke’) lacking CNS and dorsal mesoderm. Such embryos are typically seen after UV-treatment and microinjection of *BMP4* or *BMP7* mRNA into *Xenopus* embryos. These N-terminal mutations exacerbate the ventralizing activity of overexpressed wild-type xTsg in *Xenopus* (Oelgeschläger et al., 2000).

Fig. 6. xTsgW67G overexpression prevents dorsalization by LiCl. (A) Untreated stage 30 embryo. (B) Radially dorsalized embryo obtained after LiCl treatment at the 32-cell stage (DAI=9.25, *n*=16). (C) Embryo microinjected into the marginal zone of each blastomere at the four cell stage with 80 pg xTsgW67G mRNA (DAI=1.1, *n*=11) or (D) microinjected with a total of 8 ng anti-Chordin morpholino oligos at the two-cell stage (DAI=6.4, *n*=36) prior to LiCl treatment at 32-cell stage. Note that xTsgW67G causes complete ventralization, which cannot be reversed by LiCl treatment.

1999), which would cause accumulation of inhibitory Chordin/Tsg/BMP ternary complexes (Larrain et al., 2001).

However, we now report that overexpression of wild-type xTsg in zebrafish also caused a pro-BMP phenotype in the tail region. About half of the weakly dorsalized embryos had a duplication of the ventral fin tip, visible in posterior views (see inset in Fig. 7B). These duplications are typically seen in a weak allele of *chordino*, *ms2*, and in *mercedes/ogon* ventralized mutants (Hammerschmidt et al., 1996a; Wagner and Mullins, 2002). Perhaps at later stages of development, when the tail is patterned, higher Tolloid levels favor the pro-BMP activity of xTsg mRNA. A duplicated ventral fin has been recently reported for *Smad5* mutant fish (Kramer et al., 2002), making less clear whether duplication of the ventral fin always reflects a pro-BMP effect.

Whereas wild-type xTsg overexpression may cause both dorsalization and ventralization in zebrafish, hyperventralizing xTsgW67G and xTsgC59A constructs had only pro-BMP effects. As seen in Fig. 7E-G, the anterior CNS was hypoplastic, lacking brain and eyes in the most severe cases, and trunk somites were defective or absent. The degree of ventralization caused by xTsgW67G and xTsgC59A was more severe (Fig. 7) than the ventralization caused by complete loss-of-function of Chordin in zebrafish (Hammerschmidt et al., 1996a; Ross et al., 2001; Wagner and Mullins, 2002) or by knock-down using Chordin morpholinos in *Xenopus* (Oelgeschläger et al., 2003). When 800 pg xTsgW67G mRNA was injected into embryos resulting from *dini/+* x *dini/+* crosses (*n*=147), we recovered 14 embryos that had a *dini/dini* genotype by PCR but a ventralized phenotype stronger than that in *chordino* mutants. We used the *tt250* allele of *chordino*, which results in a null mutation (Hammerschmidt et al., 1996a; Schulte-Merker et al., 1997; Wagner and Mullins, 2002). Therefore, these experiments demonstrate that xTsgW67G can exert ventralizing effects even in the absence of Chordin protein.

Fig. 7. Overexpression of hyperventralizing xTsg constructs in zebrafish embryos inhibits dorsoanterior development. (A) Uninjected sibling. Wild-type zebrafish embryos were injected at the one-cell stage with 400 pg wild-type xTsg mRNA (B-D) or with 330 pg xTsgW67G mRNA (E-G). Embryos injected with wild-type xTsg mRNA in two independent experiments (*n*=144) displayed a range of dorsalization (as classified by Mullins et al., 1996): 13% Class 1 (not shown), 44% Class 2 (B), 17% Class 3 (C) and 24% Class 4 (D). Of the embryos displaying Class 1 or 2 dorsalizations, 51% also exhibited duplication of the terminal ventral fin (inset in B, indicated by arrowheads), suggestive of a tail ventralization in zebrafish. Of 199 embryos injected with 330 pg xTsgW67G mRNA in two independent experiments, 73% were moderately ventralized to levels comparable with *chordino* (E). 14% showed a phenotype more severe than *chordino* (F) and 3% displayed an even more ventralized phenotype (G). At higher doses (800 pg), xTsgW67G mRNA caused phenotypes stronger than *chordino* in 86.8% of embryos (*n*=91), with 18.7% of the type shown in G and 67% of the type shown in F. Note the almost complete absence of brain and trunk somites in G. Injection of 200 pg xTsgC59A mRNA into 60 embryos (not shown) also caused ventralization, with 82% appearing similar to E, and 5% resembling F.
Mutations in conserved cysteines of the C-terminal domain at positions 180 and 198 resulted in the opposite phenotype, a mild dorsalization, with enlarged head structures and shortened trunks. This phenotype indicated anti-BMP activity, which could be explained by their maintaining BMP-binding ability in combination with a loss of the ventralizing activity contained in the C-terminal domain. When the strongest ventralizing and dorsalizing mutations were combined in the same molecule, the resulting molecule had almost no activity in overexpression assays. Thus, the two conserved domains of Tsg appear to have opposing biological activities.

**The hyperventralizing Tsg mutants**

Biochemical studies showed that xTsgC59A and xTsgW67G mutants were unable to bind BMP4 but were still able to bind to full-length Chordin. The C-terminal mutations, however, were unable to bind Chordin, suggesting that their dorsalizing activity is caused exclusively by BMP binding. The strongest ventralizing mutant, xTsgW67G, bound to full-length Chordin and prevented the recruitment of BMP into a ternary complex (Fig. 4D,F).

The hyperventralizing mutants of xTsg described here are not a neomorphic activity resulting from the point mutations. This is because wild-type xTsg also has ventralizing effects in Xenopus embryos (Fig. 2B,C) (Oelgeschläger et al., 2000). Wild-type xTsg mRNA has very potent ventralizing effects (inhibition of CNS, notochord or muscle) in Xenopus embryos in which the levels of Chordin have been reduced by microinjection of chordin antisense morpholin oligos (Oelgeschläger et al., 2003).

xTsgW67G was not only able to antagonize the activity of full-length Chordin, but also that of an isolated CR module (Fig. 5). We had previously shown that CR1 was able to bind and antagonize BMP and that this binding could be competed by increasing amounts of xTsg protein without formation of ternary complexes (Larraín et al., 2001). It was assumed that xTsg acted via competition for BMP binding. However, as xTsgW67G did not bind BMP, the inactivation of the anti-BMP activity of this CR module must be achieved through a different molecular mechanism. This raises the possibility that the C-terminal domain of Tsg contains a biochemical activity able to inactivate CR domains. Although the C-terminal domain of Tsg presented some homology to bacterial nitrate reductases (narH) in blast searches, these similarities were weak and the catalytic residues not conserved. It will be important to compare this domain of Tsg to other enzymes once its three-dimensional structure is available.

The N-terminal domain could have an autoinhibitory effect on the biochemical activity of the C terminus. Autoinhibitory mechanisms by intramolecular interactions have been described for a variety of enzymes including the Src tyrosine kinase, GSK3β and ubiquitin ligases (Nguyen and Lim, 1997; Harwood, 2001; Du et al., 2002), and are commonly described as the jack-knife model. A requirement of the N-terminal domain for autoinhibition of Tsg might explain why multiple mutations in this domain have similar phenotypic effects on the resulting molecule that resides in the C terminus of the molecule.

**xTsg must regulate multiple CR-containing proteins**

The ventralizing (pro-BMP) activity of xTsgW67G was specific for proteins containing CR modules, as it was able to block Chordin and an isolated CR-domain, but did not inhibit the structurally unrelated BMP antagonist Noggin or a dominant-negative BMP receptor (tBR). Biochemical analyses have so far failed to detect stable binding of xTsg or xTsgW67G to isolated CR domains (Oelgeschläger et al., 2000; Larraín et al., 2001) (M.O. and E.M.D.R., unpublished). The ventralizing activity of xTsgW67G mRNA is very potent, for it can almost completely eliminate formation of CNS and dorsal mesoderm when overexpressed in Xenopus or zebrafish embryos. In addition, xTsgW67G completely blocked the dorsalizing effects of LiCl and Activin protein.

The effects of xTsgW67G cannot be solely due to inactivation of endogenous Chordin protein. In zebrafish embryos, overexpression of hyperventralizing Tsg mutants (Fig. 7) resulted in embryos that were much more ventralized than a chordin null allele (Hammerschmidt et al., 1996a; Schulte-Mmerker et al., 1997; Wagner and Mullins, 2002). Experiments using chordin mutant zebrafish embryos confirmed that xTsgW67G can exert ventralizing effects that are independent of the presence of Chordin. Taken together, these results indicate that hyperventralizing xTsg mutants must act on other CR-containing proteins in addition to Chordin.

Chordin is probably only the tip of the iceberg. CR-containing proteins are part of a growing family of secreted proteins (Garcia-Ardu et al., 2002), many of which have been shown to bind BMP. For example, CTGF and isofoms of procollagens contain single CR modules and have been shown to bind both BMPs and TGFβs (Abreu et al., 2002; Zhu et al., 1999; Larraín et al., 2000). Kielin, CRIM1 (cysteine-rich motor neuron 1), Crossveinless 2, Neurlin 1/Veurotropin and Neurulin 2 contain multiple CR repeats and have been implicated in the regulation of BMP and TGFβs (Matsui et al., 2000; Kolle et al., 2000; Larrain et al., 2000; Coffinier et al., 2001; Nakayama et al., 2001; Sakuta et al., 2001; Coffinier et al., 2002). Any of these CR-containing proteins, or as yet undiscovered ones, may contribute to dorsoventral patterning in the course of normal development.

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