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Induction of dorsal mesoderm by soluble, mature Vg1 protein

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SUMMARY

Mesoderm induction during *Xenopus* development has been extensively studied, and two members of the transforming growth factor-β family, activin βB and Vg1, have emerged as candidates for a natural inducer of dorsal mesoderm. Heretofore, analysis of Vg1 activity has relied on injection of hybrid Vg1 mRNAs, which have not been shown to direct efficient secretion of ligand and, therefore, the mechanism of mesoderm induction by processed Vg1 protein is unclear. This report describes injection of *Xenopus* oocytes with a chimeric activin-Vg1 mRNA, encoding the pro-region of activin βB fused to the mature region of Vg1, resulting in the processing and secretion of mature Vg1. Treatment of animal pole explants with mature Vg1 protein resulted in differentiation of dorsal, but not ventral, mesodermal tissues and dose-dependent activation of both dorsal and ventrolateral mesodermal markers. At high doses, mature Vg1 induced formation of 'embryoids' with a rudimentary axial pattern, head structures including eyes and a functional neuromuscular system. Furthermore, truncated forms of the activin and FGF receptors, which block mesoderm induction in the intact embryo, fully inhibited mature Vg1 activity. To examine the mechanism of inhibition, we have performed receptor-binding assays with radiolabeled Vg1. Finally, follistatin, a specific inhibitor of activin βB which is shown not to block endogenous dorsal mesoderm induction, failed to inhibit Vg1. The results support a role for endogenous Vg1 in dorsal mesoderm induction during *Xenopus* development.

Key words: *Xenopus*, mesoderm induction, Vg1, activin receptor, FGF receptor, BMP receptor, follistatin

INTRODUCTION

At the blastula stage of *Xenopus* development, vegetal blastomeres produce signals that induce mesoderm formation in overlying equatorial cells (Nieuwkoop, 1969a; Sudarwati and Nieuwkoop, 1971). Much effort has been directed at the molecular analysis of mesoderm induction and, consequently, a number of molecules with mesoderm-inducing or patterning activities have been identified. Among the described mesoderm-inducing factors, three endogenous secreted proteins, activin and bone morphogenetic protein (BMP) (members of the TGF-β family), and fibroblast growth factor (FGF), are implicated in the formation of dorsal, ventral and ventrolateral mesoderm, respectively (reviewed by Kessler and Melton, 1994). Expression of dominant inhibitory activin, FGF or BMP receptors, lacking the intracellular kinase domains, supports the involvement of these factors in endogenous mesoderm induction and patterning (Hemmati-Brivanlou and Melton, 1992; Amaya et al., 1991; Suzuki et al., 1994; Graff et al., 1994). Additional molecules, noggin, chordin, and members of the wnt family, can alter the dorsoventral pattern of mesoderm while lacking intrinsic mesoderm-inducing activity (reviewed by Kessler and Melton, 1994; Sasai et al., 1994). Despite the potent activities observed for these molecules, each fails to fulfill a strong expectation for an endogenous mesoderm inducer: localization to the vegetal pole blastomeres that are responsible for mesoderm induction and patterning during normal development. Localization of maternal activin, BMP or FGF protein has yet to be observed (Asashima et al., 1991; Fukui et al., 1993; Nishimatsu et al., 1993; Ueno et al., 1992).

Vg1 is a maternal mRNA localized to the vegetal pole of *Xenopus* eggs and early embryos (Rebagliati et al., 1985; Weeks and Melton, 1987; reviewed by Vize and Thomsen, 1994). Vg1 mRNA is synthesized early in oogenesis and becomes tightly localized to the vegetal cortex by the end of oogenesis (Melton, 1987; Mowry and Melton, 1992; Yisraeli and Melton, 1988) and, therefore, Vg1 mRNA and protein become partitioned within vegetal pole blastomeres (inducing tissue) in the early embryo (Dale et al., 1989; Tannahill and Melton, 1989). TGF-β-like proteins are synthesized as inactive precursors, which form disulfide-linked dimers and are proteolytically cleaved, releasing a mature C-terminal bioactive dimer (Massagué et al., 1994). However, endogenous Vg1 protein accumulates as an unprocessed precursor (46×10^3 M_r) and little or no mature Vg1 (18×10^3 M_r) has been detected (Dale et al., 1989; Tannahill and Melton, 1989; Thomsen and Melton, 1993). Consistent with this observation, injection of embryos with Vg1 mRNA produces high levels of Vg1 precursor, but no processed protein and, consequently, neither mesoderm induction nor developmental effects are observed (Dale et al., 1989, 1993; Tannahill and Melton, 1989; Thomsen and Melton, 1993). In contrast, injection of activin mRNA directs efficient production of mature protein and animal pole

explants are induced to form mesoderm (Thomsen et al., 1990). Therefore, although Vg1 localization suggests that it is an in vivo mesoderm-inducing signal, an inability to produce processed protein has hindered analysis of mature Vg1 activity.

Recent studies using chimeric BMP-Vg1 molecules have demonstrated mesoderm-inducing activity for mature Vg1 (Thomsen and Melton, 1993; Dale et al., 1993). A fusion protein, consisting of the N-terminal pro-region of BMP and the C-terminal mature region of Vg1, was shown to direct precursor processing, and permitted an analysis of mature Vg1 function. Injection of BMP2-Vg1 mRNA, incorporating the BMP2 tetrabasic cleavage site, resulted in induction of dorsal mesoderm (muscle and notochord) in animal pole explants and complete dorsal axis formation in ultraviolet-ventralized embryos, demonstrating the inductive activity of the Vg1 hybrid (Thomsen and Melton, 1993). In contrast, a BMP4-Vg1 hybrid, maintaining the Vg1 tetrabasic cleavage site, weakly induced mesoderm and axis formation (Dale et al., 1993).

While processing is enhanced by the hybrid constructs, the secretion of soluble, mature Vg1 by BMP4-Vg1-injected oocytes is inefficient (Dale et al., 1993), bringing into question the mechanism of processed Vg1 action in injected embryos. Specifically, it is possible that hybrid Vg1 molecules act intracellularly, or that they inhibit translation or processing of other endogenous TGF-βs, such as BMPs. Furthermore, injection of hybrid mRNAs does not allow the analysis of competence, mesoderm induction versus dorsalization, specific activity and receptor-binding specificity. In an attempt to elucidate the activity of mature Vg1 and its role during normal development, we have undertaken the production of soluble, mature Vg1.

Utilizing a hybrid activin βB-Vg1 molecule, expressed in *Xenopus* oocytes, we have obtained efficient secretion of soluble, biologically active, processed Vg1. Soluble mature Vg1 strongly induces dorsal mesoderm in animal pole explants, resulting in the formation of 'embryoids' displaying a rudimentary axial pattern. In addition, both a truncated activin type II receptor and a truncated FGF receptor fully inhibited the inducing activity of mature Vg1, while a truncated BMP receptor did not. Receptor-binding studies indicated that, although the truncated activin receptor can block Vg1 function, it does not bind Vg1 ligand. Moreover, follistatin, an inhibitor of activin, which we show is unable to block endogenous dorsal mesoderm formation, failed to inhibit mature Vg1. Our results establish the dorsal mesoderm-inducing activity of secreted Vg1 ligand and suggest that inhibition of endogenous mesoderm induction by truncated receptors (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992) may be due, in part, to an inhibition of signaling by endogenous Vg1.

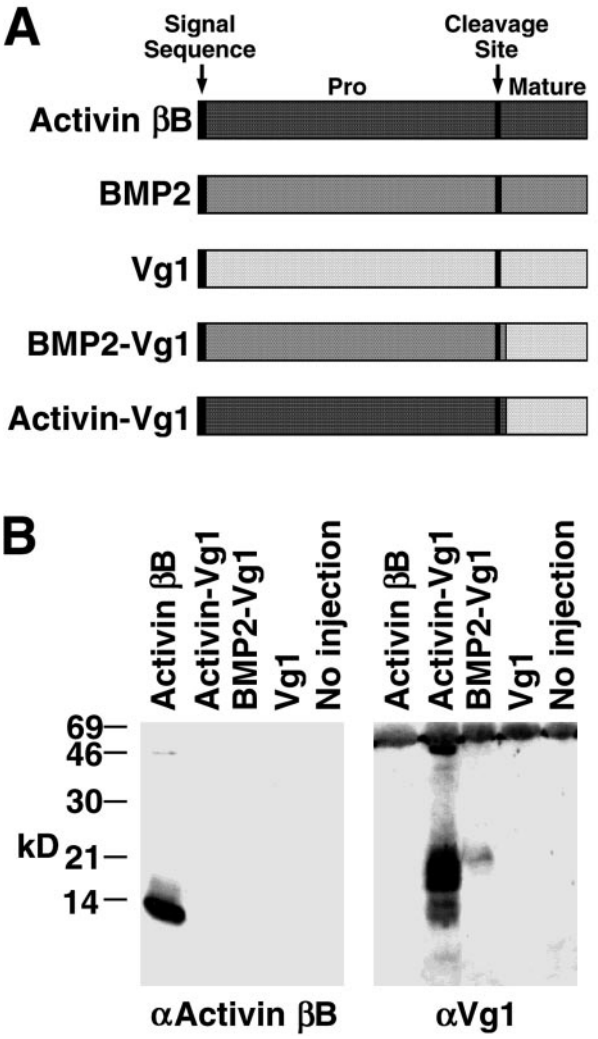

Fig. 1. (A) Chimeric Vg1 constructs. Schematic representation of activin βB, BMP2, Vg1, and chimeric BMP2-Vg1 and activin-Vg1. Members of the TGF-β superfamily, these genes contain an signal sequence, pro-region, tetrabasic cleavage site and mature region. Activin βB and BMP2, but not Vg1, form disulfide-linked dimers that are subsequently cleaved, releasing the mature C-terminal peptide as a secreted bioactive dimer. The chimeric constructs, fused four amino acids downstream of the cleavage site, are designed to facilitate processing and secretion of mature Vg1. (B) Western blot analysis of oocyte supernatants. Oocytes were injected with the indicated mRNA and conditioned supernatants (10 μl) analyzed by western blotting of reducing SDS-PAGE using activin-specific (left) or Vg1-specific (right) antisera. Activin βB mRNA directs secretion of mature protein. While Vg1 mRNA directs no secretion and BMP2-Vg1 directs low levels of secretion, activin-Vg1 mRNA results in secretion of abundant mature Vg1 protein.

MATERIALS AND METHODS

Embryological procedures and histology

Xenopus eggs were fertilized, microinjected and cultured using standard procedures (Thomsen and Melton, 1993). For animal cap experiments, embryos were injected at the 2-cell stage, animal cap explants prepared at stage 8 and cultured in 0.5× MMR supplemented with 50 ng/ml recombinant basic FGF (Gibco/BRL) or oocyte-conditioned media. For marginal zone experiments, dorsal or ventral explants were prepared from stage 10.25 embryos (Nieuwkoop and Faber, 1967) and cultured in 0.5× MMR supplemented with oocyte-conditioned media. Oocytes were surgically removed from anesthetized females, follicle cells removed by digestion with 2 mg/ml collagenase (Sigma, type IA) in OR2 (5 mM Tris-HCl (7.8), 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4) (Peng, 1991), injected with 50 ng of capped, in vitro transcribed RNA and cultured at 19°C in OR2 supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 mg/ml BSA. Oocyte-conditioned media was prepared by incubating oocytes in OR2 with $MgCl_2$, $CaCl_2$ and BSA (10 μl/oocyte) in 96-well microtiter

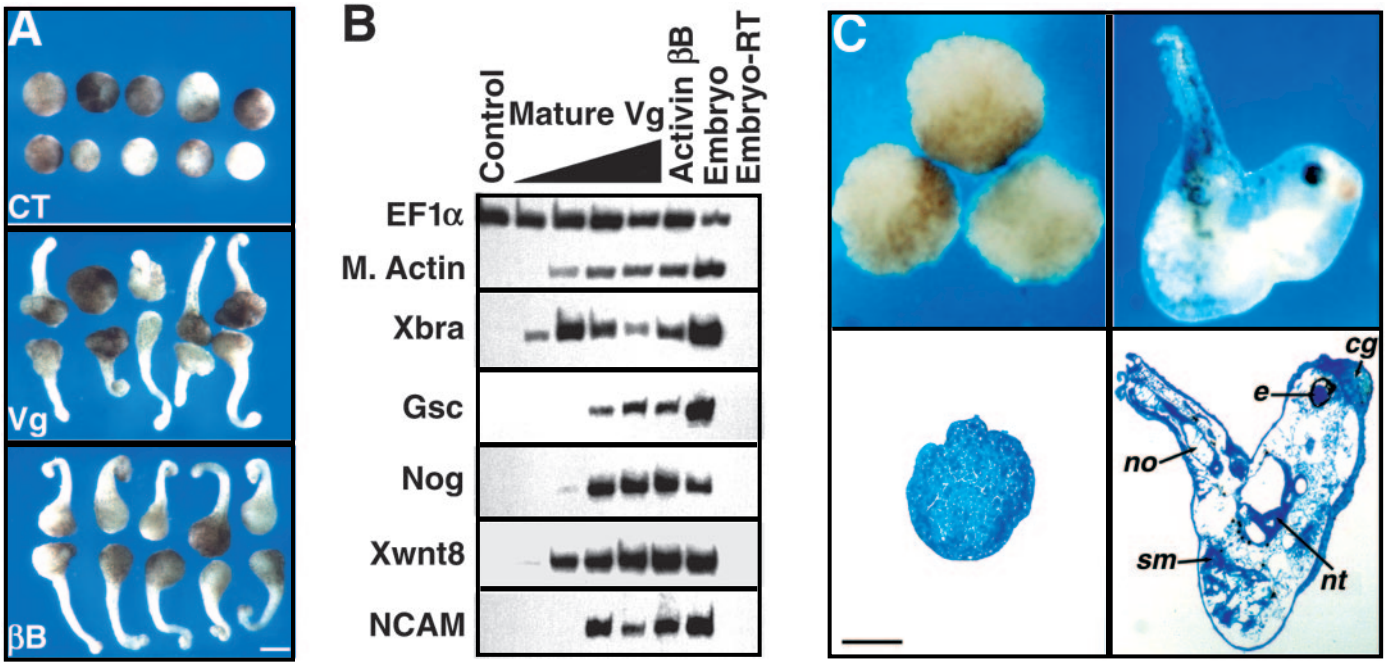

Fig. 2. (A) Mature Vg1 induces morphogenetic movements in ectodermal explants. Blastula-stage animal pole explants were treated with 10% supernatant (1/10 dilution) until the gastrula stage and cultured to the neurula stage (stage 15). Mature Vg1 (Vg) results in a dramatic elongation indicative of mesoderm induction (Symes and Smith, 1987). Activin βB (βB) results in similar elongation, while supernatant of uninjected oocytes (CT) have no effect. Scale bar, 300 μm. (B) Dose-dependent induction of mesodermal markers by mature Vg1. Blastula-stage animal pole explants were treated with increasing doses of mature Vg1 supernatant (1%, 3%, 10%, 30%), or control (30%) or activin βB supernatant (10%), cultured to the neurula stage and analyzed by RT-PCR. At low doses (1%) the general marker Xbra is induced; at intermediate doses (3%) the ventrolateral marker Xwnt8 and the dorsolateral marker cardiac actin (M. Actin) are induced; and at high doses (10-30%) the dorsoanterior markers goosecoid (Gsc) and noggin (Nog), and the neural marker NCAM are induced. Activin βB treatment results in a similar response and control supernatants have no effect. EF1α is a loading and reverse transcription control and the embryo and embryo-RT are additional positive and negative controls. (C) Induction of embryoids by mature Vg1. Blastula animal pole explants treated with a high dose of mature Vg1 and cultured to the late tadpole stage (stage 40), differentiated into embryoids displaying a rudimentary axial organization, with anterior-posterior pattern and head structures. The embryoid shown (top right) has a clear head-to-tail pattern and pigmented eye and cement gland. A histological section (bottom right) reveals differentiated notochord (no), somitic muscle (sm), neural tube (nt), eye (e) and cement gland (cg). Treatment with supernatant of uninjected oocytes has no effect and explants form atypical epidermis (left). Scale bar, 150 μm.

plates. Media was conditioned for 3-4 days and stored at 4°C. For histology, samples were fixed in Bouin's fixative and processed for Paraplast sectioning and Giemsa staining.

Construction of activin βB-Vg1 hybrid and mRNA synthesis

The activin βB-Vg1 hybrid was constructed by PCR amplification of a fragment of a *Xenopus* activin βB gene (Thomsen et al., 1990) encoding the N-terminal pro-region, the tetrabasic cleavage site and the first four amino acids of the C-terminal mature region. This fragment was ligated, in frame, to a PCR amplified fragment of the Vg1 gene (Weeks and Melton, 1987) encoding the C-terminal mature region lacking the first four amino acids. The ligation product was reamplified, digested at terminal restriction sites and cloned into a derivative of pSP64T (Krieg and Melton, 1987). The activin-Vg1 construct and nucleotide sequence are available upon request. This hybrid is analogous to the BMP2-Vg1 construct previously described (Thomsen and Melton, 1993; Fig. 1A).

For in vitro synthesis of capped mRNA, linearized templates for activin βB-Vg1, BMP2-Vg1 (Thomsen and Melton, 1993), activin βB (Sokol et al., 1991), Vg1 (Tannahill and Melton, 1989), noggin (Smith and Harland, 1992), follistatin (Hemmati-Brivanlou et al., 1994), truncated FGF receptor (Amaya et al, 1991), truncated activin receptor (Hemmati-Brivanlou and Melton, 1992), truncated BMP2/4 receptor (Graff et al., 1994) or β-galactosidase (Smith and Harland, 1991) were used to program the Megascript SP6 transcription kit (Ambion).

Western blotting and quantitation of secreted protein

Supernatants of injected oocytes were resolved by 15% reducing SDS-PAGE, electroblotted to nylon membranes and probed using a mature region-specific Vg1 monoclonal antiserum (Tannahill and Melton, 1989) or an affinity-purified activin βB polyclonal antiserum specific for the mature region (provided by P. Klein). Cross-reacting peptides were detected using alkaline phosphatase-conjugated secondary antibodies, visualized by chemiluminescence (Bio-Rad). The concentration of secreted proteins in oocyte supernatants was determined by western blotting and quantitation of serial dilutions of supernatant and a standard curve of in vitro translated Vg1 and activin. The concentration of in vitro translated proteins was calculated by measuring incorporation of [^{35}S]methionine.

Reverse transcription-polymerase chain reaction

RNA isolation, reverse transcription, PCR conditions and primer sequences have been previously described (Wilson and Melton, 1994). PCR products were resolved on 5% non-denaturing acrylamide gels. The ubiquitous EF1α mRNA (Krieg et al., 1989) was used as a RNA extraction and reverse transcription control. Xbra is a general mesodermal marker (Smith et al., 1991) and Xwnt8 is a marker of ventrolateral mesoderm (Christian et al., 1991). Cardiac actin is a

marker of somitic muscle (Stutz and Sphor, 1986) and goosecoid and noggin are markers of dorsal mesoderm (Blumberg et al., 1991; Smith and Harland, 1992). NCAM is a general marker of neural tissue (Kintner and Melton, 1987).

Activin receptor ligand-binding analysis

An epitope-tagged version of the activin type II receptor (XARmyc), containing a COOH-terminal c-Myc peptide (Evan et al., 1985), was used for receptor crosslinking studies (A. Hemmati-Brivanlou, unpublished). Defolliculated oocytes were injected with 10 ng of XARmyc mRNA and cultured for 2 days prior to binding analysis. Metabolically labeled activin and mature Vg1 was prepared as described above with the addition of [^{35}S]methionine (50 μCi/ml) to the culture medium. In a total volume of 200 μl, ten oocytes expressing XARmyc were incubated with 0.4 nanomolar labeled ligand (~10^4 cts/minute/μg), in the presence or absence of 4 nanomolar unlabeled competitor, for 30 minutes at 4°C. Oocytes were washed extensively with cold OR2 supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$ and ligand-receptor complexes chemically crosslinked with the reducible crosslinker DTSSP (2 mM; Pierce) for 30 minutes at 4°C. Following lysis in RIPA (50 mM Tris-HCl (8.0), 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS), ligand-receptor complexes were immunoprecipitated from cleared lysates (5 minutes at 5000×***g***) using the Myc-epitope specific monclonal antibody 9E10 (5 μg/ml) (Evan et al., 1985). Ligand-receptor complexes were reduced, co-precipitating ligand resolved by 15% reducing SDS-PAGE. Co-precipitating activin is released from complexes by reducing the crosslinker, and is therefore resolved as an ~14×10^3 M_r band, distinguishing the binding of mature ligand from unprocessed protein, which was present at low levels in the preparations used.

RESULTS

Secretion of processed Vg1 by *Xenopus* oocytes

Expression of hybrid BMP-Vg1 molecules in embryos directs precursor processing, but little evidence for efficient secretion of processed Vg1 is available. BMP2-Vg1 expression in COS cells (DSK, G. Thomsen and DAM, unpublished) or *Xenopus* oocytes resulted in low or undetectable levels of secreted mature Vg1 (Fig. 1A,B), although intracellular processing of precursor was apparent. BMP4-Vg1 expression in oocytes failed to direct efficient secretion of mature protein, although polyanion addition did release low levels of processed protein. The biological activity of this material is unknown since no activity assessment was reported (Dale et al., 1993). Consistent with these observations, secretion of BMP2 or BMP4 is not detected in these systems (D. S. K. and D. A. M., unpublished), suggesting that the pro-region may be a target for the regulation of secretion (Gray and Mason, 1990). In addition, these observations raise the possibility that the effects of BMP-Vg1 in embryos may not be due to secretion of mature protein, but rather to an intracellular activity of the hybrid. Alternatively, oocytes and embryos may differ in their capacity to process and secrete various members of the TGF-β family.

In contrast to the BMP-Vg1 hybrids, functional activin βB ligand is efficiently secreted in both COS cells and oocytes (Fig. 1B; Thomsen et al., 1990). Therefore, to obtain soluble mature Vg1, an additional hybrid molecule was prepared containing the activin βB signal sequence, pro-region, tetrabasic cleavage site and four amino acids of the activin βB mature region fused to the mature region of Vg1 (Fig. 1A). To test the function of this activin-Vg1 hybrid, animal pole explants of injected embryos were prepared at the blastula stage, cultured and scored for mesoderm formation. Injection of activin-Vg1 mRNA resulted in tissue elongation and expression of muscle-specific cardiac actin, similar to that observed with BMP2-Vg1 injection (data not shown). Induction by the activin-Vg1 hybrid was not due to expression of the activin pro-region since an activin-BMP2 hybrid induced expression of ventral, not dorsal mesodermal markers, consistent with the activity of BMP2 (data not shown).

Defolliculated oocytes were injected with 50 ng of in vitro transcribed RNA and cultured for 3 days. Conditioned supernatants were analyzed by western blotting using antisera specific for the mature region of activin βB or Vg1 (Fig. 1B). Injection of activin βB mRNA resulted in secretion of processed protein, detected as a reduced monomer of ~14×10^3 M_r. No secreted mature Vg1 was detected following injection of native Vg1 mRNA, and a small amount of mature Vg1 secretion was directed by BMP2-Vg1. Injection of activin-Vg1 mRNA resulted in secretion of abundant mature Vg1, detected as a series of bands of ~18×10^3 M_r. The different mature species presumably reflect varying glycosylation states, as described for the endogenous precursor (Tannahill and Melton, 1989; Dale et al., 1989, 1993). The ability of activin-Vg1 to direct secretion of mature protein was comparable in efficiency to activin βB and, in both cases, a lesser amount of secreted precursor (~46×10^3 M_r) is also detected. The concentration of mature Vg1 and activin in oocyte-conditioned medium was determined by western blotting and quantitation against a standard curve of Vg1 or activin protein (see Materials and Methods). In a typical experiment, 200 oocytes were injected, producing approximately 2 ml of supernatant containing soluble, processed Vg1 and activin βB at concentrations of 100-500 ng/ml.

Mature Vg1 induces dorsal mesoderm in animal pole explants

Oocyte supernatants were tested for mesoderm-inducing activity on animal pole explants. Blastula-stage animal poles (prospective ectoderm) were explanted, incubated with supernatant, cultured to the neurula stage and scored for the formation of mesodermal tissues and expression of mesodermal markers. Supernatants of uninjected, Vg1- or BMP2-Vg1-injected oocytes failed to induce morphogenetic movements or expression of mesodermal markers (Fig. 2A,B; data not shown). Supernatants of activin-Vg1- or activin βB-injected oocytes strongly induced morphogenetic movements indicative of mesoderm induction, expression of mesodermal markers and differentiation of the mesodermal tissues, muscle and notochord (Fig. 2A-C).

Low doses of mature Vg1 induced expression of the general mesodermal marker Xbra (Smith et al., 1991). Intermediate doses induced the ventrolateral marker Xwnt-8 (Christian et al., 1991) and the dorsalateral marker cardiac actin (Stutz and Sphor, 1986). High doses of mature Vg1 induced expression of the dorsal mesodermal markers goosecoid (Blumberg et al., 1991) and noggin (Smith and Harland, 1992), and, by secondary induction, the neural marker NCAM (Kintner and Melton, 1987). These markers were also induced by activin βB, which was a positive control, but not by supernatant of uninjected oocytes (Fig. 2B). Globin, a definitive marker of ventral mesoderm was not induced by Vg1 or activin (data not shown).

Induction of morphogenetic movements in Vg1-treated explants was consistent with the response profile of mesodermal markers. At low doses (Xbra positive), no elongation was apparent and, at higher doses (cardiac actin positive), marked elongation is observed (data not shown). Using this preparation of mature Vg1, the lowest dose that induces mesoderm is approximately 1 ng/ml or 40 pM. The specific activity of mature Vg1 is similar to that found for activin βB and other members of the TGF-β family (Vale et al., 1990).

Examination of mature Vg1-treated explants cultured to the late neurula stage by immunohistochemistry and histology revealed the differentiation of mesodermal tissues, notochord and somitic muscle, as well as neural tissue (data not shown). Explants cultured to the late tadpole stage, following treatment with mature Vg1, often developed a high degree of axial organization. These 'embryoids' displayed a rudimentary anterior-posterior pattern with organized head structures, including eyes, and a functional neuromuscular system, similar to results with activin protein (Sokol et al., 1990). Histological sections showed the presence of muscle, notochord, neural tissue and differentiated eye (Fig. 2C).

Inhibiton of mature Vg1 activity by the truncated activin and FGF receptors

To address the relation between mature Vg1 and endogenous mesoderm-inducing signals, we tested the ability of dominant inhibitory receptors to block the activity of mature Vg1. A truncated form of an activin type II receptor (tAR), lacking the intracellular kinase domain, blocked formation of detectable mesoderm and expression of mesodermal markers in embryos (Hemmati-Brivanlou and Melton, 1992). Animal pole explants of embryos injected with 4 ng of tAR mRNA were treated with mature Vg1 or activin βB. tAR expression fully inhibited induction of morphogenetic movements and expression of brachyury and cardiac actin by mature Vg1. As expected, tAR also blocked activin βB activity and resulted in NCAM expression regardless of treatment with supernatants (Table 1). This confirms similar results reported by Schulte-Merker et al. (1994). A dominant inhibitory FGF receptor (tFGFR), lacking the kinase domain, blocked formation of trunk and posterior mesoderm, resulting in loss of axial mesoderm and tail structures (Amaya et al., 1991). In addition to blocking FGF activity, tFGFR also blocks mesoderm induction by activin βB in animal pole explants (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994). Injection of 4 ng of tFGFR mRNA resulted in a complete block of mesoderm induction by both mature Vg1 and activin βB (Table 1; Schulte-Merker et al., 1994). These observations suggest that the ability of the truncated activin and FGF receptors to perturb mesoderm induction in the embryo may, in fact, be due to an inhibition of endogenous Vg1 signaling. In contrast, animal cap expression of a truncated BMP2/4 receptor (tBR), capable of converting ventral mesoderm to dorsal mesoderm in embryos (Suzuki et al., 1994; Graff et al., 1994), failed to inhibit the mesoderm-inducing activity of mature Vg1 (Table 1). In these experiments tFGFR and tBR blocked induction by FGF and BMP2, respectively (data not shown).

Table 1. Mesoderm induction by mature Vg1 is blocked by truncated activin and FGF receptors

	βGal	tAR	tFGFR	tBR
Control	–	–	–	–
Mature Vg1	+	–	–	+
Activin βB	+	–	–	+

At the 2-cell-stage embryos were injected with 4 ng of a truncated activin type II receptor (tAR), a truncated FGF receptor (tFGFR), a truncated BMP2/4 receptor (tBR) or β-galactosidase (βGal) mRNA. Blastula-stage animal pole explants ($n \geq 10$) were prepared and treated with mature Vg1, activin βB or control supernatants (10%). Following culture to the neurula stage, mesoderm induction was assessed by explant elongation and expression of brachyury and cardiac actin, as was done for the experiments shown in Fig. 5. Induction by mature Vg1 was fully inhibited by the truncated activin and FGF receptors, similar to their effects on activin. No inhibition was detected with the truncated BMP receptor, or the control β-galactosidase injection. These results were obtained in three independent experiments.

The activin type II receptor is not a high affinity receptor for mature Vg1

The inhibition of mature Vg1 activity by the truncated activin receptor suggested that the activin type II receptor (XAR1) may also function as a Vg1 receptor. This possibility was examined by binding analysis of radiolabeled ligands (see Methods) to an epitope-tagged version of XAR1 (XARmyc) expressed in oocytes. Ligand binding was assessed by competition studies using chemical crosslinking and immunoprecip-

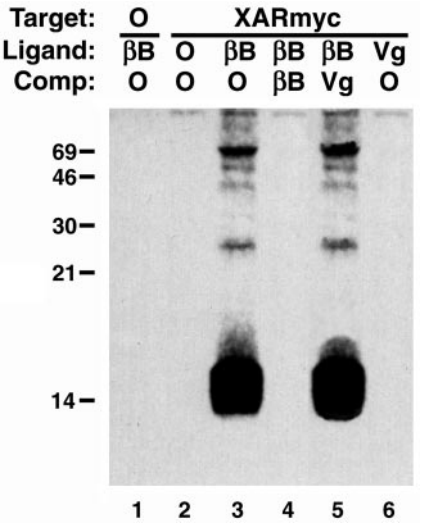

Fig. 3. Mature Vg1 is not bound by the activin type II receptor. Uninjected oocytes, or oocytes expressing a myc-tagged activin type II receptor (XARmyc) were bound with radiolabeled activin βB or mature Vg1, complexes chemically crosslinked, immunoprecipitated with a myc-specific antiserum and visualized by 15% reducing SDS-PAGE and fluorography. Co-precipitating activin is released from complexes by reducing the crosslinker, and is therefore resolved as an ~14×10^3 M_r band. Uninjected oocytes did not bind radiolabeled activin βB (lane 1) and XARmyc-expressing oocytes displayed no binding in the absence of radiolabeled ligand (lane 2). Receptor-expressing oocytes strongly bound activin (lane 3) and this binding was competed by a 10-fold excess of unlabeled activin (lane 4). In contrast, a 10-fold excess of unlabeled mature Vg1 was unable to compete activin binding (lane 5). Furthermore, a 10-fold higher dose of radiolabeled Vg1 than that required for activin binding did not result in detectable binding (lane 6). The higher molecular weight bands (lanes 3, 5) may be due to non-reduced ligand-receptor complexes and co-precipitating unprocessed activin.

itation. While radiolabeled activin βB was not bound by control oocytes, expression of XARmyc resulted in strong binding of activin, which was competed by a 10-fold excess of unlabeled activin (Fig. 3). Although a complete competition of activin binding is apparent in this experiment, quantitation of additional experiments shows an approximately 7-fold reduction of activin binding by a 10-fold excess of unlabeled activin. In contrast, radiolabeled Vg1 showed no detectable binding to XARmyc, even at a 10-fold higher dose than that required for activin binding. Furthermore, an excess of unlabeled mature Vg1 was unable to compete for activin binding. These observations suggest that the activin type II receptor is not a Vg1 receptor, and that inhibition of Vg1 signaling by the truncated activin receptor is due to an interaction with an endogenous Vg1-specific receptor.

Follistatin does not block mature Vg1 activity or endogenous dorsal mesoderm induction

A natural inhibitor of activin function is the activin-binding protein follistatin (Kogawa et al., 1991; Nakamura et al., 1990). *Xenopus* follistatin is maternally expressed and can block activin induction of animal pole explants (Tashiro et al., 1991; Fukui et al., 1993, 1994; Hemmati-Brivanlou et al., 1994). It has been reported that, unlike the truncated activin receptor, rat follistatin has no effect on the early embryo (Slack, 1991; Schulte-Merker et al., 1994). To examine more closely the effect of follistatin expression on normal development, *Xenopus* follistatin mRNA (XFS-319; Hemmati-Brivanlou et al., 1994) was injected into both blastomeres at the 2-cell stage. In contrast to previous reports (Schulte-Merker et al., 1994), we do observe an effect of follistain on early development. At the tadpole stage, XFS overexpression results in a consistent loss of posterior structures and an apparent enhancement of dorsoanterior structures (Fig. 4A,B). As assessed by both histology and molecular markers, no suppression of dorsal mesoderm is detected in response to XFS. Even at high doses of XFS mRNA, differentiated dorsal mesodermal tissues (notochord and muscle) are present in injected embryos (Fig. 4C,D), consistent with the maintenance of dorsal mesodermal marker expression at the gastrula stage (Fig. 4E). These observations indicate that XFS does not inhibit endogenous induction of dorsal mesoderm and do not support a role for activin in the formation of dorsal mesoderm in vivo.

To examine further the relation of mature Vg1 to endogenous mesoderm induction, the ability of follistatin to inhibit mature Vg1 activity was examined. Mature Vg1 or activin supernatants were combined with an equal volume of XFS supernatant prior to addition to blastula animal pole explants. At these doses, XFS fully blocked activin-induced morphogenetic movements and expression of cardiac actin, whereas mature Vg1 activity was unaffected (Fig. 5). Even at a dose of follistatin 100-fold higher than that required to inhibit activin, no inhibition of mature Vg1 was observed (data not shown).

Mature Vg1 does not dorsalize gastrula ventral mesoderm

At the gastrula stage the organizer produces a signal that can alter the fate of lateral mesodermal tissues, resulting in formation of mesodermal tissues with more dorsal character (Dale and Slack, 1987; Slack and Foreman, 1980; Yamada, 1950). While neither activin nor FGF display this dorsalizing activity (Slack et al., 1992), soluble noggin protein, expressed in the organizer (Smith and Harland, 1992), can induce muscle in gastrula ventral marginal zone explants (Smith et al., 1993). We tested the dorsalizing activity of mature Vg1 by incubating blastula or gastrula ventral marginal zone explants in supernatants containing mature Vg1, activin βB, or noggin and examined the expression of cardiac actin. Mature Vg1, activin and noggin treatment resulted in expression of muscle-specific cardiac actin in blastula ventral marginal zone explants.

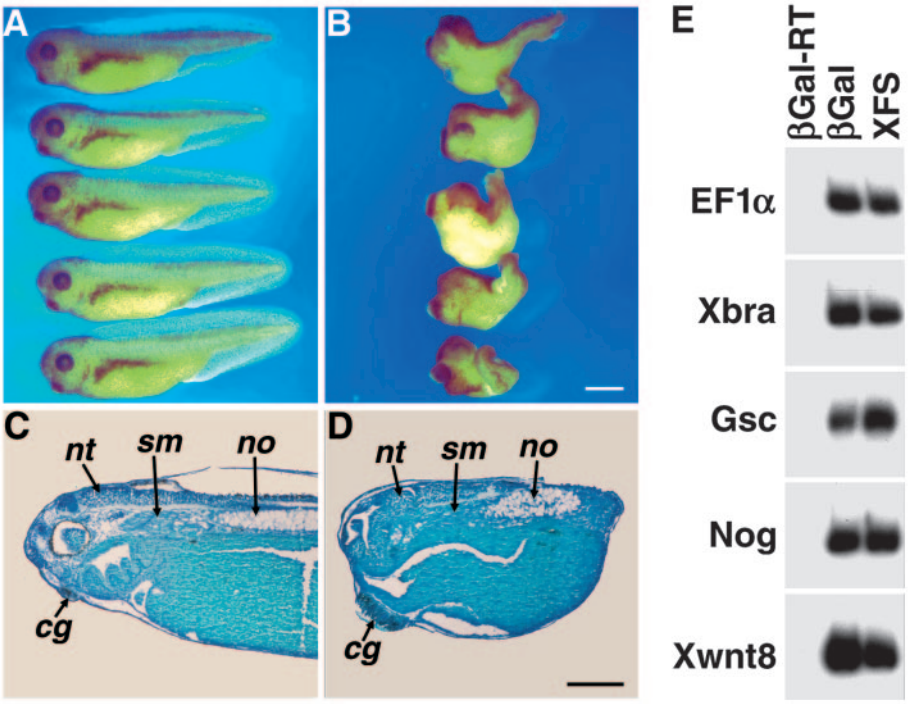

Fig. 4. Follistatin does not inhibit endogenous dorsal mesoderm induction. Both blastomeres of 2-cell-stage embryos were injected with β-galactosidase mRNA (A) or follistatin mRNA (B) (1 ng total mRNA) and cultured to the tadpole stage. Follistatin resulted in a loss of posterior structures with maintenance or enhancement of dorsoanterior structures. Histological analysis reveals the presence of dorsal mesodermal tissues in both β-galactosidase (C)- and follistatin (D)-injected embryos (no, notochord; sm, somitic muscle; nt, neural tube; cg, cement gland). (E) Follistatin does not inhibit expression of mesodermal markers at the gastrula stage. 2-cell-stage embryos were injected with a total of 2 ng of β-galactosidase (βGal) or follistatin (XFS) mRNA, harvested at the mid-gastrula stage (stage 11) and analyzed by RT-PCR. The expression of a general mesodermal marker, brachyury (Xbra), the dorsal markers, goosecoid (Gsc) and noggin (Nog), and a ventrolateral marker, Xwnt8, were unaffected by follistatin. EF1α is a loading and reverse transcription control. Scale bars, 1 mm (A,B) and 0.5 mm (C,D).

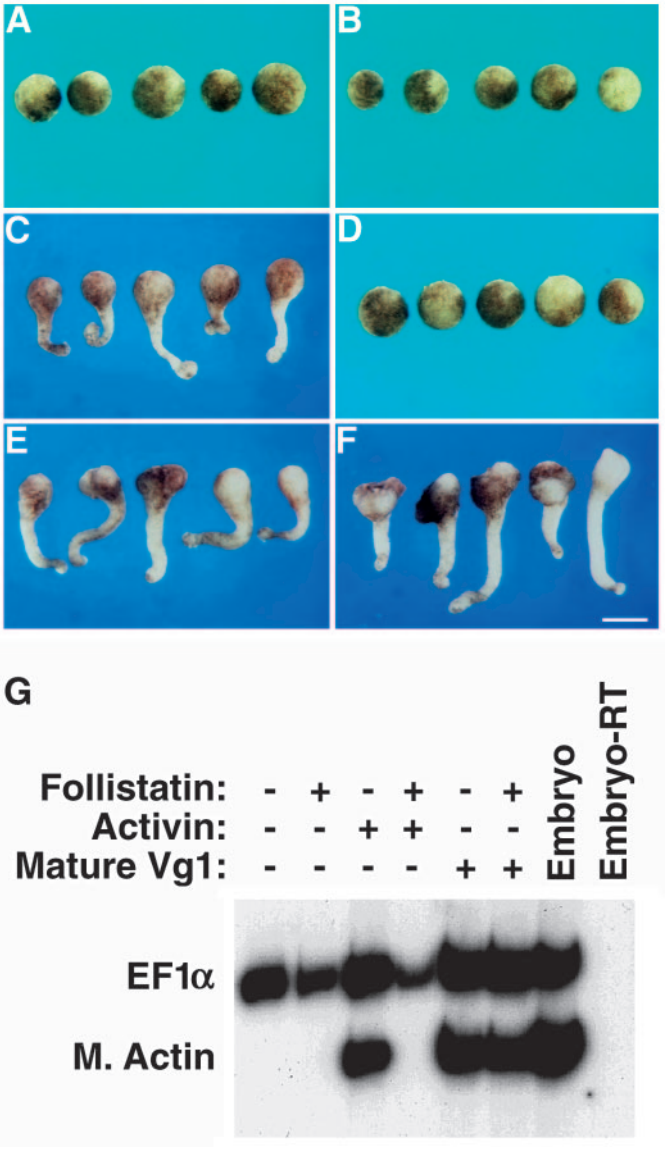

Fig. 5. Follistatin does not inhibit mature Vg1 activity. Blastula-stage animal pole explants were treated with control (A,B), activin βB (C,D), or mature Vg1 (E,F) supernatants following mixture and preincubation with control (A,C,E) or follistatin (B,D,F) supernatants. While follistatin fully blocks activin-induced morphogenetic movements, induction by mature Vg1 was unaffected. (G) Follistatin fails to inhibit cardiac actin (M. Actin) induction by mature Vg1. Blastula animal pole explants were treated with mature Vg1 or activin only, or with these supernatants preincubated with follistatin. Upon reaching the neurula stage, samples were analyzed by RT-PCR. While follistatin fully inhibits cardiac actin induction by activin, no effect on mature Vg1 activity is detected. EF1α is a loading and reverse transcription control. Scale bar, 300 μm.

However, neither mature Vg1 nor activin induced muscle actin expression in gastrula stage ventral marginal zone explants, while noggin-treated explants and control dorsal marginal zone explants expressed substantial levels of cardiac actin (Fig. 6). The inability of mature Vg1 to dorsalize the gastrula ventral marginal zone is consistent with the loss of competence to respond to mesoderm induction at the gastrula stage (Jones and

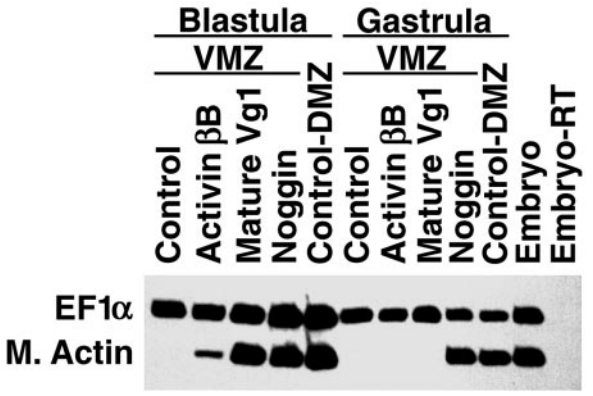

Fig. 6. Mature Vg1 does not dorsalize gastrula ventral marginal zone explants. Dorsal or ventral marginal zone explants were prepared from blastulae (stage 8) or early gastrulae (stage 10.25) and incubated in supernatants (30%) containing activin, mature Vg1 or noggin, or in a supernatant of uninjected oocytes (control). At the neurula stage, samples were analyzed by RT-PCR. While all three factor induced cardiac actin (M. Actin) in blastula ventral marginal zone explants, only noggin did so in gastrula-stage explants. EF1α is a loading and reverse transcription control.

Woodland, 1987), and is an expected characteristic of a natural mesoderm inducer.

DISCUSSION

We report the preparation of soluble, biologically active mature Vg1 by expression of an activin βB-Vg1 chimera in *Xenopus* oocytes. The results demonstrate that the mesoderm-inducing activity of Vg1 does reside in the secreted mature region, establishing the mechanism of action of injected BMP-Vg1 hybrids (Dale et al., 1993; Thomsen and Melton, 1993). Soluble, mature Vg1 is a potent inducer of dorsal mesoderm when applied to animal pole explants, resulting in expression of dorsal markers (goosecoid, noggin and cardiac actin), a ventrolateral marker (Xwnt8) and a general mesodermal marker (Xbra), but not the ventral mesodermal marker globin. Differentiation of mesodermal tissues (muscle and notochord) and secondary induction of neural tissue is observed and, in many cases, explants form a well-organized axial pattern, including head structures. Based on dosage and degree of response, it can be concluded that mature Vg1 and activin βB have markedly similar activities on animal pole explants. Furthermore, we find a precise correspondence between the inhibition profile of mature Vg1 and that of endogenous dorsal mesoderm induction. Truncated forms of the activin and FGF receptors, which interfere with endogenous mesoderm induction, fully inhibit mesoderm induction by mature Vg1. In contrast, a truncated BMP receptor, which stimulates formation of dorsal mesoderm, does not inhibit mature Vg1. Additional experiments demonstrate that follistatin, an inhibitor of activin function, does not block endogenous dorsal mesoderm induction, and fails to block mature Vg1 activity. In addition to supporting a role for Vg1 in dorsal mesoderm induction, these results argue against the involvement of endogenous activin in dorsal mesoderm induction. In sum, our observations support a role for processed Vg1 in natural dorsal mesoderm

induction and indicate that the inhibitory effects of truncated receptors may reflect an inhibition of Vg1 signaling in the developing embryo.

Our experiments differ from previous studies of follistatin in several ways (Slack, 1991; Schulte-Merker et al., 1994). In these previous studies, an alternative splice product of the rat follistatin gene was used and this has reduced activity compared to the *Xenopus* gene (Fukui et al., 1993). Rat follistatin failed to display any biological activity at low doses, such as the neuralizing activity previously described for XFS (Hemmati-Brivanlou et al., 1994), and high doses resulted in embryo death. In addition, it is not clear if a sufficient excess of follistatin was used in the analysis of Vg1 inhibition (Schulte-Merker et al., 1994). The studies presented here utilized *Xenopus* follistatin that is active and non-toxic, even at high doses. In addition, the described preparations of concentrated, soluble mature Vg1 and XFS allowed the use of a large excess of XFS in inhibition studies, conclusively demonstrating the inability of follistatin to inhibit mature Vg1.

Overexpression of follistatin in embryos and animal pole explants can stimulate neural markers, but inhibition of endogenous mesoderm formation has not been reported (Hemmati-Brivanlou et al., 1994; Schulte-Merker et al., 1994). In contrast, our observations indicate that follistatin can block development of posterior structures, suggesting a potential role in the patterning of mesoderm. An examination of the ability of follistatin to stimulate dorsal mesoderm formation is now underway. In addition, a dose of follistatin 100-fold higher than that required to inhibit activin does not inhibit the mesoderm-inducing activity of mature Vg1. The failure of follistatin to block either mature Vg1 or endogenous induction of dorsal mesoderm is consistent with the function of endogenous Vg1 in dorsal mesoderm induction.

The mechanism of mature Vg1 inhibition by the truncated activin type II receptor is not clear. Results of receptor-binding analysis indicate that mature Vg1 does not bind to the activin type II receptor with high affinity. The TGF-β-related receptor family is a diverse group of related molecules that form heterodimeric complexes (Massagué et al., 1994), and inhibition of signaling by overexpression of a truncated receptor could result from inactivation of an associated subunit within an existing or novel receptor complex. Therefore, the activin type II receptor could be a subunit of a Vg1 receptor complex or have no involvement in an endogenous Vg1 receptor complex.

A model for the role of Vg1 in dorsal mesoderm formation

An oriented cortical rotation during the first cell cycle establishes initial dorsoventral polarity in *Xenopus* (Elinson and Rowning, 1988; Vincent and Gerhart, 1987). The dorsoventral pattern is reflected in the capacity of dorsal vegetal cells to induce notochord and muscle, while ventral vegetal cells induce mesenchyme, blood and small amounts of muscle (Nieuwkoop, 1969b; Boterenbrood and Nieuwkoop, 1973; Dale and Slack, 1987). In addition, as early as the 32-cell stage, dorsal vegetal blastomeres can organize a complete body axis following transplantation to the ventral vegetal region (Gimlich and Gerhart, 1984; Kageura, 1990). The dorsal vegetal cells that constitute this organizing center (Nieuwkoop center) have an endodermal fate and induce dorsal mesoderm, resulting in formation of Spemann's organizer and axial organization (Elinson and Kao, 1989; Gerhart et al., 1989).

It is proposed that cortical rotation directs the localized processing of Vg1 precursor, resulting in formation of the Nieuwkoop center and, subsequently, the Spemann organizer. The proteolytic activation of Vg1 could be dependent on a locally activated protease or restricted release of a cleavage site inhibitor. It has been shown that ventral vegetal injection of ultraviolet-ventralized embryos with BMP2-Vg1 results in complete axial rescue and injected cells exclusively populate the endoderm, consistent with the formation of a Nieuwkoop center (Thomsen and Melton, 1993). While both noggin and Xwnt-8 injection result in axial rescue and Nieuwkoop center formation (Smith and Harland, 1991, 1992; Sokol el al., 1991), these factors, unlike Vg1, are not localized to vegetal pole blastomeres during normal development and do not induce dorsal mesoderm. Vg1 is a promising candidate for an endogenous factor required for Nieuwkoop center formation. The induction of dorsal mesoderm in explants by mature Vg1 may dependent on the prior formation of an endodermal organizing center. Consistent with this suggestion would be the induction of endodermal markers by mature Vg1 and the detection of endodermal tissues in apposition to mesodermal tissues in induced explants. These predictions are currently being tested. It should be noted that the induction of an endoderm-specific antigen by mesoderm-inducing factors has been reported (Jones et al., 1993), consistent with the proposed expectations.

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