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Endoderm induction by the organizer-secreted factors chordin and noggin in *Xenopus* animal caps

Yoshiki Sasai[1], Bin Lu, Stefano Piccolo and Eddy M.De Robertis[2]

Howard Hughes Medical Institute and Department of Biological Chemistry, University of California, Los Angeles, CA 90095-1737, USA

[1]Present address: Department of Biological Sciences, Kyoto University Faculty of Medicine, Yoshida-Konoe, Sakyoku, Kyoto 606, Japan

[2]Corresponding author

Y.Sasai and B.Lu contributed equally to this work

Spemann's organizer has potent neural inducing and mesoderm dorsalizing activities in the *Xenopus* gastrula. A third activity, the organizer's ability to induce a secondary gut, has been difficult to analyze experimentally due to the lack of early gene markers. Here we introduce *endodermin*, a pan-endodermal gene marker, and use it to demonstrate that chordin (Chd), a protein secreted by the organizer region, is able to induce endodermal differentiation in *Xenopus*. The ability of *chd*, as well as that of *noggin*, to induce endoderm in animal cap explants is repressed by the ventralizing factor BMP-4. When FGF signaling is blocked by a dominant-negative FGF receptor in *chd*-injected animal caps, neural induction is inhibited and most of the explant is induced to become endoderm. The results suggest that proteins secreted by the organizer, acting together with known peptide growth factors, regulate differentiation of the endodermal germ layer.

Keywords: BMP-4/chordin/endodermin/FGF/Spemann's organizer

Introduction

Recent molecular studies on the amphibian organizer have revealed many factors acting at distinct steps during early dorsoventral patterning of the embryo. The Spemann organizer is formed in the dorsal marginal zone in response to signals emanating from cells in the dorsovegetal region of the embryo, called the Nieuwkoop center. The Nieuwkoop center signals, whose activity can be mimicked by some TGF-β family molecules such as activin and Vg-1 (Cooke *et al.*, 1987; Thomsen and Melton, 1993), induce several organizer-specific transcription factors: *goosecoid* (Cho *et al.*, 1991), *Xnot-1* and *-2* (Gont *et al.*, 1993; von Dassow *et al.*, 1993), *Xlim-1* (Taira *et al.*, 1992) and *HNF-3β* (Dirksen and Jamrich, 1992). The organizer tissue, once established, patterns the mesoderm and ectoderm by secreting extracellular factors: noggin (Smith and Harland, 1992), follistatin (Hemmati-Brivanlou *et al.*, 1994) and chordin (Chd) (Sasai *et al.*, 1994). An interesting recent observation is that all these secreted organizer factors can dorsalize mesoderm of the marginal zone and can induce neural tissues in animal cap explants (Smith and Harland, 1992; Lamb *et al.*, 1993; Sasai *et al.*, 1995). Thus, mesoderm dorsalizing factors and neural inducers are two sides of the same coin; the signals appear to be the same and the different responses depend on the target tissue (De Robertis and Sasai, 1996). The activities of noggin, follistatin and Chd are antagonized by the ventralizing factor BMP-4 (Sasai *et al.*, 1995), which is expressed in the complementary region to that of synthesis of the organizer factors during gastrulation (Fainsod *et al.*, 1994; Schmidt *et al.*, 1995a).

This antagonistic system of dorsoventral patterning signals has been conserved during evolution. In *Drosophila*, *decapentaplegic* (*dpp*) is a morphogen promoting dorsal development (Ferguson and Anderson, 1992a; Wharton *et al.*, 1993), while *short-gastrulation* (*sog*) promotes ventral development by antagonizing *dpp* (Zusman *et al.*, 1988; Ferguson and Anderson, 1992b; François *et al.*, 1994). *Drosophila dpp* and *sog* are structural and functional homologs of *BMP-4* and *chd* respectively. Vertebrate *BMP-4* (and its close relative *BMP-2*) can rescue the *dpp* mutation (Padgett *et al.*, 1993) and *dpp* has a ventralizing activity in frog (Holley *et al.*, 1995). Microinjection of *sog* mRNA into *Xenopus* embryos shows dorsalizing activity, as does *chd* mRNA (Holley *et al.*, 1995; Schmidt *et al.*, 1995b). The expression pattern of *dpp/BMP-4* and *sog/chd* in *Drosophila* and vertebrates supports the idea that the dorsoventral axes of vertebrates and arthropods were inverted during the course of evolution, as first proposed by Geoffroy Saint-Hilaire in 1822 (reviewed in De Robertis and Sasai, 1996).

Despite much progress in the analysis of mesodermal and neural induction in the vertebrate embryo, little is known about the molecular mechanisms by which the endodermal germ layer is generated. One indication that endoderm formation can result from inductive cell–cell interactions comes from the classical organizer experiment (Spemann and Mangold, 1924). By grafting a piece of tissue from the dorsal lip, they showed that a secondary gut was formed in the twinned embryonic axis. Although 70 years have elapsed since this experiment, endodermal induction remains one of the least understood aspects of amphibian development. Here we introduce a pan-endodermal marker, *endodermin*, which enabled us to show that *chd* and *noggin* are potent inducers of endoderm in animal cap explants and that this activity is counteracted by BMP-4. When the neural differentiation pathway is inhibited by injection of a dominant-negative FGF receptor (Amaya *et al.*, 1991), most of the animal cap tissue injected with *chd* mRNA becomes endodermal. We conclude that proteins secreted by the organizer, such as Chd and noggin, previously known to pattern the ectoderm and mesoderm (Lamb *et al.*, 1993; Sasai *et al.*, 1995), can also induce differentiation of the endodermal germ layer.

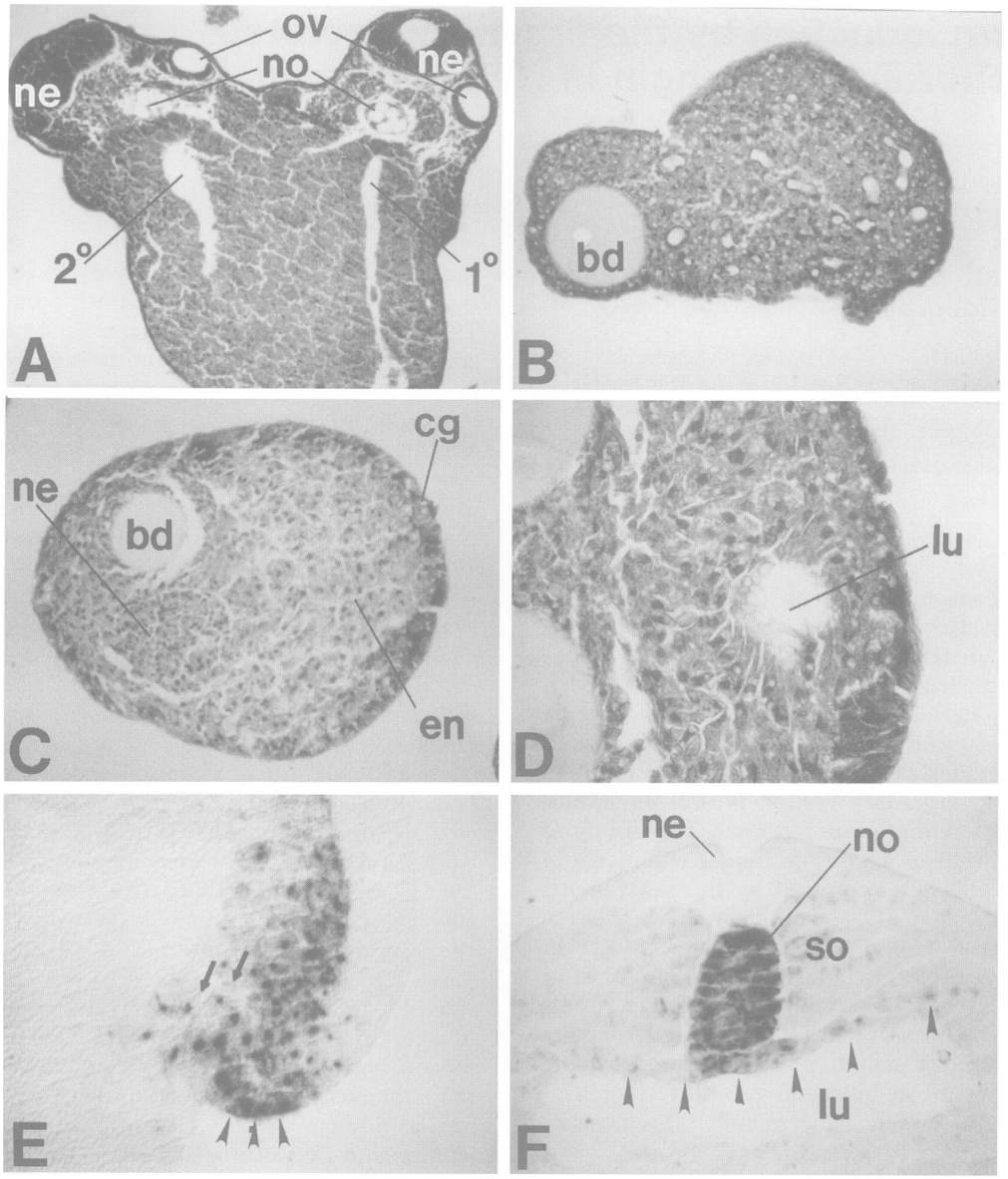

Fig. 1. Chd induces endoderm differentiation and activates the pan-endodermal marker *endodermin*. (**A**) Twinned axes caused by ventral injection of *chd* mRNA (300 pg) contain a secondary gut cavity (2°). no, notochord; ne, neural tube; ov, otic vesicle. (**B**) An agarose bead (bd) containing control medium conditioned by cells infected with AcNPV baculovirus (Pharmingen) sandwiched between animal cap explants; the tissue remains as atypical epidermis. (**C**) A bead containing *chd* conditioned medium induces cement gland (cg), neural tissue (ne) and a mass of endoderm-like cells (en) in the animal caps. (**D**) Higher power view of an explant as in (C) in which a gut-like lumen (lu) surrounded by cylindrical epithelial cells developed. (**E**) *In situ* hybridization showing that at the gastrula stage endogenous *chd* RNA is expressed in endoderm precursor cells located on the surface layer of the dorsal lip (arrowheads) and in the bottle cells of the blastopore (arrows). (**F**) At late neurula (stage 18) *chd* RNA is expressed mostly in the notochord (no), but some endogenous expression remains in the dorsal aspect of the endoderm (arrowheads). Ventral endoderm is negative. ne, neural plate; so, somite; lu, archenteron lumen.

Results

Induction of endoderm-like cells by Chd

During the course of experiments testing the neural inducing activity of Chd protein, we noticed the formation of endoderm-like tissue in animal caps. This observation was of interest because at about the same time we noted that *in vivo* the injection of *chd* mRNA leads to the formation of a secondary gut in the twinned body axis (Figure 1A), indicating that *chd* can mimic the gut inducing activity of Spemann's organizer. The Chd protein used in the initial animal cap experiments was obtained as a soluble protein of 120 kDa in baculovirus conditioned medium that precipitated when dialyzed into amphibian saline. To concentrate Chd protein, conditioned medium was bound to chromatography beads which were then placed between two gastrula animal caps and cultured (see Materials and methods). Control medium from a culture infected with a baculovirus lacking the *chd* insert had no effect on the explants, which differentiated into atypical epidermis (Figure 1B). When beads containing Chd were used, in addition to the expected neural and cement gland tissues (Sasai *et al.*, 1995), a different kind of differentiation was induced by Chd beads in these sandwiches (Figure 1C). Blocks of eosinophilic cells with large cytoplasms were formed. These cells resemble endoderm (see for example Figure 1A), even though animal cap cells contain less yolk. In some cases the endoderm-like cells formed cavities

surrounded by cylindrical epithelial cells with basal nuclei and apical differentiations reminiscent of the embryonic gut lumen (Figure 1D). Similar tissue was subsequently noted in caps injected with *chd* mRNA or DNA, which provided a purer system than the conditioned medium and was used in the rest of this study.

chd is expressed in dorsal endoderm precursors

In view of these results, we performed *in situ* hybridizations and histological analyses to determine whether endogenous *chd* is expressed at the right time and place to function in endodermal differentiation. At the gastrula stage strong *chd* expression was observed in the surface layer of the dorsal lip and in the bottle cells (Figure 1E), which are fated to form pharyngeal and dorsal gut endoderm (Nieuwkoop and Florschütz, 1950; Keller, 1975; Shih and Keller, 1992). At the late neurula stage, in addition to the previously described notochordal expression (Sasai *et al.*, 1994), transcripts were detectable in the cytoplasm and nuclei (arrowheads in Figure 1F) of dorsal endoderm. The nuclear expression suggests that *chd* may continue to be transcribed in dorsal endoderm at the neurula stage. We conclude that the expression pattern of *chd* is consistent with a possible role in the differentiation of the dorsal aspect of embryonic endoderm.

Endodermin, a pan-endodermal gene marker

An obstacle to the study of endoderm differentiation in *Xenopus* has been a lack of suitable gene markers. Endoderm is not easy to identify histologically and for this reason we failed to recognize endoderm induction in a previous study (Sasai *et al.*, 1995). A homeobox gene, *XlHbox 8*, is a useful early marker, but its expression does not cover all endodermal cells and is limited to a narrow band of foregut and pancreas (Wright *et al.*, 1988), so that absence of *XlHbox 8* does not necessarily mean absence of endoderm. Very recently a gene marker that specifically marks the small intestine, intestinal fatty acid binding protein (involved in the uptake of fatty acids), has been introduced (Henry *et al.*, 1996). A monoclonal antibody marker, 4G6, is also available, but it tends to stain the lumen of the gut at later stages of development (Jones *et al.*, 1993). We were fortunate in that during the differential screen that led to the isolation of *chd* from organizer tissue (Sasai *et al.*, 1994) we had identified a cDNA clone designated *endodermin* (*edd*). We now report the full-length sequence of the *edd* cDNA (4950 nucleotides, GenBank accession no. L63543) and that it provides a very useful early pan-endodermal marker. As shown in Figure 2, *edd* encodes a large secreted protein of 1461 amino acids that is a novel member of the α2-macroglobulin (α2-M) family of proteins that inactivate proteases via a thioester bond (Sottrup-Jensen, 1987; Vaughn and Vale, 1993; Webb *et al.*, 1994).

Expression of *edd* starts in the dorsal lip and from there extends to the entire rim of the blastopore (Figure 3A). In histological sections *edd* transcripts are strongest in the surface layer of the blastopore periphery in cells that are fated to become endoderm (Nieuwkoop and Florschütz, 1950; Keller, 1975), however, transcripts are also found in axial mesoderm precursors. At late neurula expression is found in the notochord, prechordal plate, hatching gland and the entire endoderm (Figure 3B and D). At tailbud

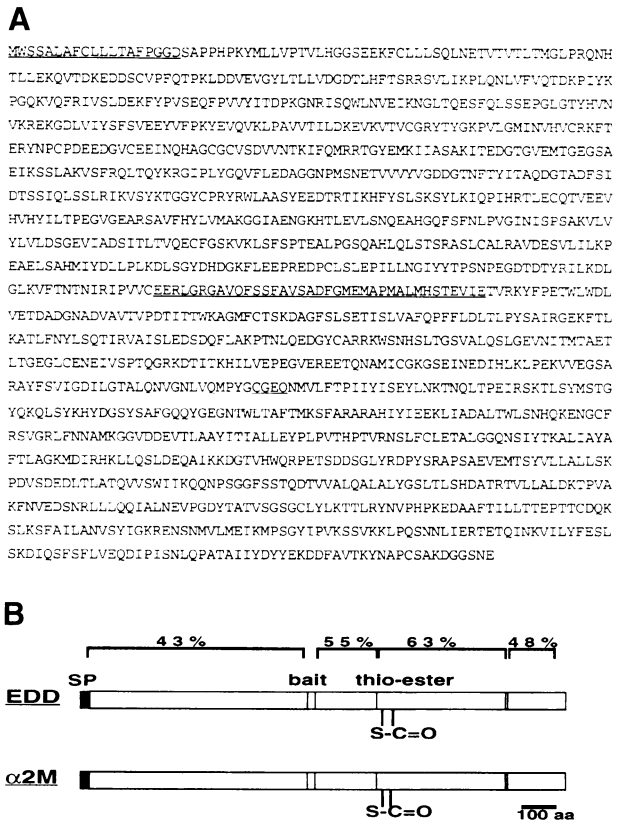

Fig. 2. The primary structure of *Xenopus* endodermin protein. (**A**) The deduced amino acid sequence of Edd (pBS-eddL6). The signal peptide (1–19), the bait region (687–723) and the thioester site (966–969) are underlined. (**B**) Protein structure of *Xenopus* Edd compared with that of the human α2-M protease inhibitor. Arrows indicate the bait region where proteases cleave α2-M and trigger covalent binding via a nearby thioester bond (Sottrup-Jensen, 1987). Percentage amino acid identities were determined by the Wisconsin GCG program (49% for the entire protein compared with human α2-M).

stages *edd* expression is very strong and localized almost exclusively to the endoderm (Figure 3C and E). We conclude that *edd* is a pan-endodermal marker at tailbud stages, although at earlier stages it also marks notochordal and prechordal mesodermal cell precursors.

chd induces edd in the embryo and in animal caps

Microinjection of *chd* mRNA into four radial sites at the 4-cell stage led to strong radial expression of *edd* in the blastopore periphery, mimicking the early dorsal expression in the gastrula (Figure 3F and G). When animal caps injected with control *lacZ* mRNA or with *chd* mRNA were explanted at the gastrula stage and cultured until siblings reached stage 30 (tailbud), patches of variable size of *edd* expression were found by *in situ* hybridization in the *chd*-injected sample (Figure 3H and I). To confirm that these patches of *edd*-positive cells corresponded to endoderm, histological sections of the *chd*-injected animal caps were performed and it was found that the *edd* patches consisted of endoderm-like cells with large cytoplasms (data not shown). The induction of endoderm in gastrula animal caps was supported by RT–PCR assays, shown in Figure 4. Injection of *chd* DNA (driven by the cytoskeletal actin, CSKA, promoter, which starts to be expressed in substantial amounts at the early gastrula stage; Lamb

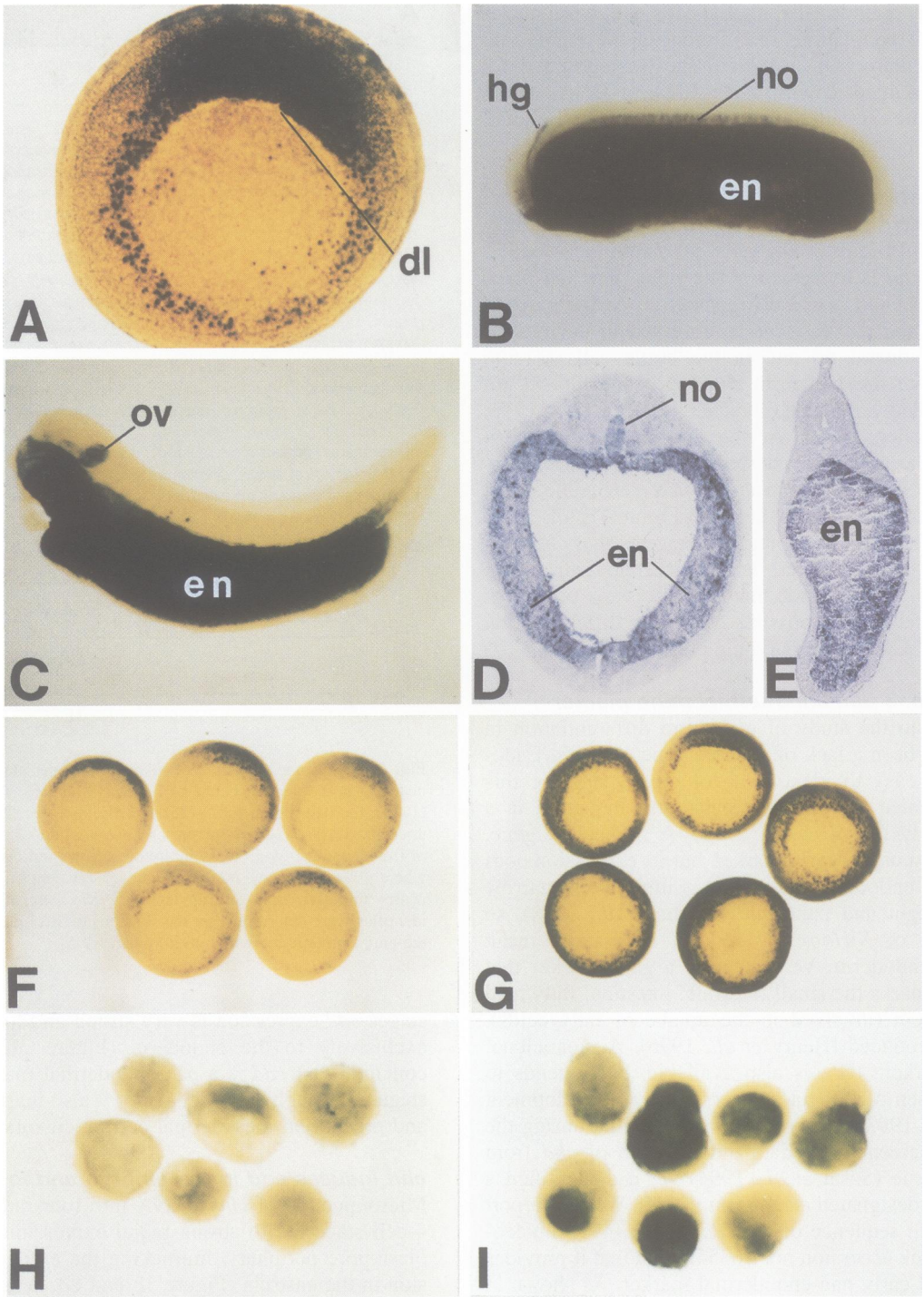

Fig. 3. Regulation of *edd* expression analyzed by *in situ* hybridization. (**A–C**) Whole mount *in situ* hybridization of *edd* at stages 11, 20 and 36 respectively. dl, dorsal lip; no, notochord; hg, hatching gland; en, endoderm; ov, otic vesicle. In (C) there is also expression in the thin hypochord located above the massively staining endoderm. (**D** and **E**) Paraffin sections of embryos hybridized with *edd* and stained with BCIP/NBT at stages 20 and 36 respectively. no, notochord; en, endoderm. (**F** and **G**) Embryos injected into each of the four blastomeres with 300 pg *lacZ* (n = 11) control or *chd* (n = 19) mRNA respectively. Note that *edd* dorsal expression becomes radial. (**H** and **I**) Animal cap explants injected with *lacZ* (n = 11) or *chd* (n = 32) mRNA respectively and cultured until stage 30. *chd* induces patches of *edd* expression.

et al., 1993) or of *chd* mRNA led to the induction of the endodermal markers *edd* and *XlHbox 8*. This induction took place in the absence of mesoderm formation, as determined by the lack of α-actin, collagen II (Figure 4) and α-globin (see Figure 6 below) expression. As reported earlier (Sasai *et al.*, 1995), neural induction (N-CAM) and cement gland formation (CG-13) are also induced by *chd*. *noggin*, like *chordin*, has dorsalizing and neural inducing activities (Lamb *et al.*, 1993). Injection of *noggin* mRNA had very similar effects to those of *chd*, leading to the expression of *edd* and *XlHbox 8* (Figure 4).

The endodermal and neural inductions caused by *chd* and *noggin* were repressed by injection of CSKA-BMP4, a *BMP-4* expressing plasmid (Figure 4). This suggested

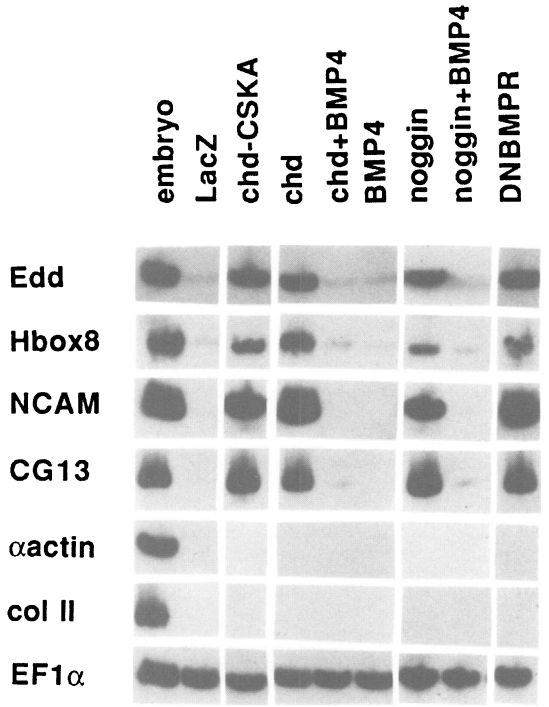

Fig. 4. *chd*, *noggin* and DNBMPR induce endodermal markers in animal caps. In these RT–PCRs endoderm formation (*edd*, *XlHbox 8*) is accompanied by induction of neural (N-CAM) and cement gland markers (CG-13), but takes place in the absence of mesoderm induction (α-actin, collagen II). Elongation factor 1α (EF1α) was used as a loading control. Lanes contained RNA from whole embryos at stage 30 or from animal caps injected with: control *lacZ* mRNA, pCSKA-*chd* DNA, *chd* mRNA, *chd* mRNA and pCSKA-Bmp4, pCSKA-Bmp4, *noggin* mRNA, *noggin* mRNA and CSKA-Bmp-4 DNA or DNBMPR mRNA.

that endogenous BMP signals (Fainsod *et al.*, 1994) might repress endodermal as well as neural (Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Xu *et al.*, 1995) differentiation in gastrula animal caps. In agreement with this possibility, when endogenous BMP signaling was blocked by injection of a dominant-negative BMP receptor (DNBMPR) (Graff *et al.*, 1994; Suzuki *et al.*, 1994), endodermal and neural differentiation took place (Figure 4). We conclude that the organizer-specific factors Chd and noggin can promote endodermal as well as neural differentiation in *Xenopus* animal caps and that their activity can be counteracted by BMP-4. Endogenous BMP signaling would function to prevent endoderm differentiation in animal caps.

FGF signaling is required for neural but not for endodermal induction by chd

To obtain a system in which *chd* induced predominantly the formation of endoderm (rather than of neural tissue), we manipulated the FGF signaling pathway. It has recently been shown that FGF can repress the expression of *XlHbox 8* in *Xenopus* endodermal explants (Gamer and Wright, 1995). It has also been shown that bFGF can induce neural tissues in animal caps that have been transiently disaggregated or treated in medium containing very low calcium and magnesium (Kengaku and Okamoto, 1993, 1995; Lamb and Harland, 1995) and that FGF can posteriorize anterior neural tissue (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). We therefore tested whether endodermal differentiation can take place in animal caps when the FGF signaling pathway is blocked. Injection of a *Xenopus* FGF dominant-negative receptor mRNA (XFD) (Amaya *et al.*, 1991) alone did not change the differentiation of animal caps, which formed atypical epidermis (Figures 5A and 6). However, when co-injected with *chd* mRNA, XFD inhibited neural induction and endoderm became the predominant differentiated tissue (Figures 5B and C and 6). Conversely, when gastrula animal caps (which are no longer competent for mesoderm induction) from embryos injected with *chd* mRNA were treated with soluble bFGF, endodermal differentiation was reduced and most of the cells in the explant adopted a neural fate (Figure 5D). In agreement with a previous report for *noggin* (Lamb and Harland, 1995), the neural tissue induced by *chd* in the presence of FGF was of a more posterior character than that formed by *chd* alone (Figure 6), as indicated by the up-regulation of the hindbrain marker *Krox-20* and of the floor plate marker *F-spondin* (Ruiz i Altaba *et al.*, 1993) and by histological analyses in which CNS structures of both anterior (eyes) and more posterior (hindbrain-like with a large ventricle and a thin roof) origin were detected (Figure 5D). We conclude that FGF signaling is required for neural but not for endodermal induction in animal caps. In the presence of the FGF dominant-negative receptor *chd* induces mostly endoderm in these explants.

Discussion

In this study the *edd* marker was used to reveal the endoderm-inducing activities of the organizer-secreted proteins Chd and noggin and the counteracting action of BMP-4. These factors have been implicated in dorsoventral patterning of ectoderm and mesoderm (reviewed in De Robertis and Sasai, 1996) and are now proposed to play a role in the endodermal germ layer as well. Chd and noggin may explain in part the induction of secondary guts by Spemann organizer grafts (Spemann and Mangold, 1924).

Endodermin as a pan-endodermal marker gene

The *edd* gene, introduced here, is a pan-endodermal marker for all endodermal differentiation. At tailbud stages *edd* is expressed in the entire endoderm, but at earlier stages it is detected also in the notochord. Therefore, *edd* serves as a good marker for endoderm if it is used at the tailbud stages. *edd* encodes a large secreted protein with significant homology to α2-M family protease inhibitors. There are many α2-M class molecules reported in mammals (Sottrup-Jensen, 1987) and the highest level of sequence identity to endodermin is found with human α2-M (49%) and with murinoglobulin (46%). α2-M family members have been implicated not only in protease inactivation, but also in the serum complement cascade (three of the complement components are α-2M family members) and in the binding of growth factors such as TGF-β2 and activin (Vaughn and Vale, 1993; Webb *et al.*, 1994). Because a cascade of proteases plays a role in the establishment of dorsoventral polarity in *Drosophila* and because some α2-M-like proteins can bind TGF-βs, a possible role for *edd* in embryonic patterning is an

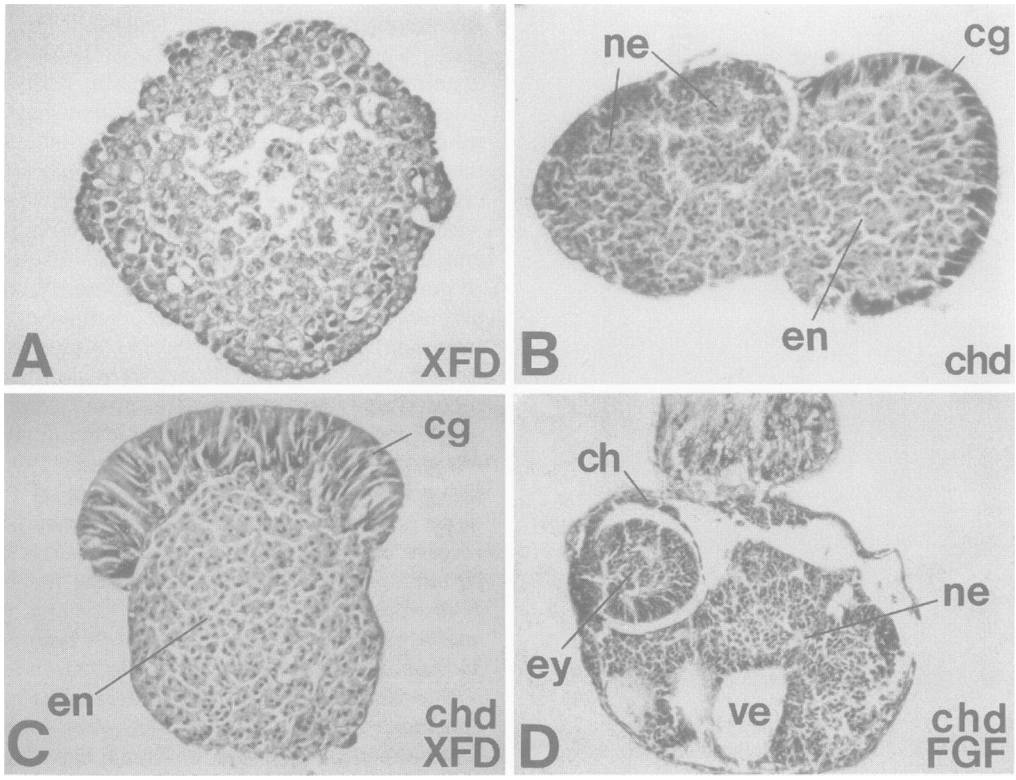

Fig. 5. (**A**) Histological appearance of atypical epidermis in an animal cap injected with FGF dominant-negative receptor *XFD* (Amaya *et al.*, 1991) (n = 25) and cultured until the third day, when siblings reached stage 42. The histological appearance of XFD-injected caps was indistinguishable from that of *lacZ*-injected explants (data not shown). (**B**) Animal cap injected with *chd* mRNA, showing neural, cement gland and endodermal differentiation (n = 28). (**C**) Animal caps co-injected with *chd* and *XFD* mRNAs differentiate into large masses of endoderm-like tissue and neural differentiation is inhibited (n = 50). (**D**) Animal caps injected with *chd* mRNA and treated with soluble bFGF at stage 10.75 develop anterior (eyes, ey; choroid, ch) and more posterior neural structures (containing ventricular, ve, cavities reminiscent of hindbrain), but little endoderm (n = 70).

intriguing subject for future studies. However, we have so far failed to find strong biological activities for *edd*, except that ventral induction of *edd* mRNA causes formation of a bulge on the side of the embryo at low frequencies.

A possible in vivo role for chd in endodermal differentiation

Histological analysis of *chd* mRNA distribution revealed that *chd* is expressed not only in the axial mesoderm but also in the endoderm, such as the superficial layer of the dorsal lip, the bottle cells of the blastopore and the archenteron roof. The expression pattern of *chd* in the *Xenopus* embryo (Figure 1E and F) suggests that Chd should mimic the formation of dorsal (Figure 7), rather than ventral, endoderm in these animal cap experiments. However, it is difficult to assign regional characteristics to the induced endoderm because this would require markers specific for ventral and for dorsal endoderm that are unavailable at present. Since *XlHbox 8* is induced by *chd* and *noggin*, the endodermal tissues in the injected caps must contain some foregut endoderm (Wright *et al.*, 1988; Gamer and Wright, 1995), which is predominantly of dorsal origin (Nieuwkoop and Florschütz, 1950; Keller, 1975; Shih and Keller, 1992).

Are signals from the organizer, such as Chd and noggin, required for all endodermal differentiation? Two experiments suggest that this is not the case: first, *edd* is expressed in ventral marginal zone explants and, second, *edd* is expressed in grade 0 UV-ventralized embryos (data not shown). Thus, the pan-endodermal marker *edd* can be expressed in the complete absence of an organizer. It is reasonable to assume that in these experimental situations the endoderm formed is of the ventral type. We assume, but have not proven, that the endoderm induced by Chd and noggin in animal caps is of the dorsal type.

It is of interest that the effects caused by injection of *chd* and *noggin* mRNA on neural, cement gland and endodermal induction can be mimicked by blocking the endogenous BMP signaling pathway with a dominant-negative BMP receptor (Figure 4). In addition, overexpression of *BMP-4* can antagonize the effects of *chd* and *noggin* on mesodermal, neural and endodermal differentiation. The induction of endoderm by Chd and its antagonism by BMP-4 can be caused by injection of DNA expression constructs under the control of the CSKA promoter. Since this promoter is not expressed until early gastrulation (Lamb *et al.*, 1993), this indicates that the signaling events described here take place during gastrulation, the stage at which the archenteron (gut cavity) is formed. Further support for the action of Chd at gastrulation or later is provided by the ability of Chd protein beads to induce endoderm in gastrula animal cap sandwiches (Figure 1C).

Taken together, the findings further extend the model (Figure 7) that the organizer is the source of signals (*chd*, *noggin* and others) that act to antagonize ventralizing signals provided by *BMP-4* and other factors during

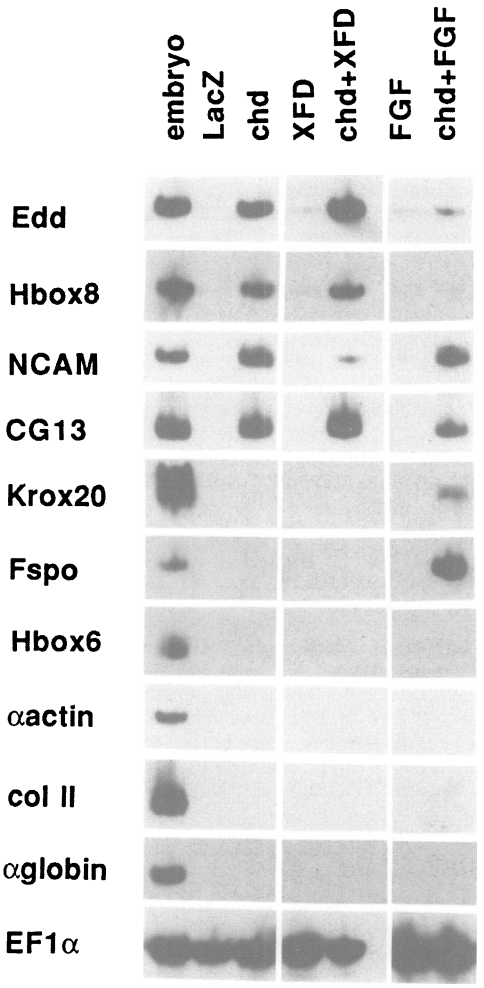

Fig. 6. FGF signaling is required for neural differentiation but not for endoderm induction. In *chd*-injected animal caps a dominant-negative FGF receptor, XFD, suppresses neural (N-CAM) but not endodermal (*edd*, *XlHbox 8*) nor cement gland (CG-13) formation. Consequently, the proportion of cells adopting an endodermal fate increases in these explants (Figure 5C). Addition of bFGF significantly reduces induction of endodermal markers by *chd*, but not the neural marker N-CAM. The deuterencephalic markers Krox-20 (rhombomeres 3 and 5) and F-spondin (floor-plate of midbrain and CNS posterior to it) were induced by FGF together with *chd*, but the spinal cord marker *XlHbox 6* (*Hoxb-9*) was not (formation of spinal cord by FGF was found to be very dependent on the stage of the animal caps by previous workers; 18). All these inductions took place in the absence of mesoderm formation, marked by collagen II (notochord), α-actin (somite) and α-globin (ventral mesoderm). RNAs used for RT–PCR were from whole embryos or from animal caps injected with: *lacZ* mRNA, *chd* mRNA, XFD mRNA, *chd* and XFD mRNA, bFGF protein or *chd* mRNA and bFGF protein.

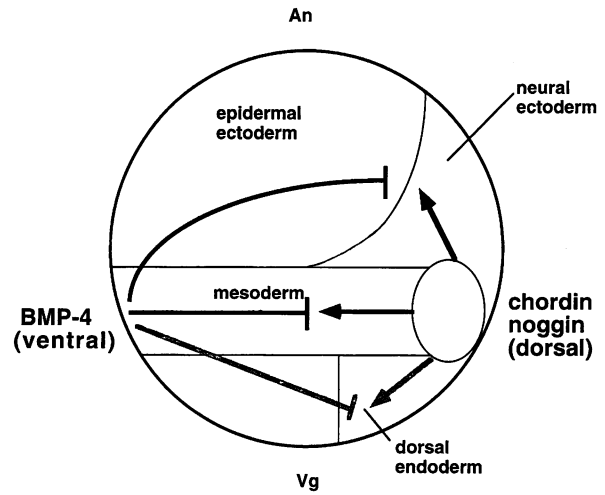

Fig. 7. A model of how the antagonistic signals involving organizer factors (Chd and noggin) and the ventralizing signals (BMP-4 and others) might regulate the differentiation of the three germ layers. In addition to the previously known neural induction in the ectoderm and dorsalization in the mesoderm (black arrows), the organizer signals can induce endodermal tissues (gray arrow) which express the pan-endodermal marker *edd* and the foregut marker *XlHbox 8*. Since the foregut is mostly of dorsal origin and Chd is expressed in the dorsal archenteron, we believe that this induction reflects the formation of dorsal, but not ventral, endoderm (see Discussion). The ventralizing factor BMP-4 can counteract the activities of the organizer signals in all three germ layers.

gastrulation (De Robertis and Sasai, 1996). Endogenous *BMP-4* functions to repress neural development in the ectoderm (Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995; Xu *et al.*, 1995), dorsal development in the mesoderm (Graff *et al.*, 1994; Suzuki *et al.*, 1994; Steinbeisser *et al.*, 1995) and endoderm differentiation in animal caps (this study). As depicted in Figure 7, the present results suggest that the organizer-secreted factors Chd and noggin promote the formation of dorsal endoderm, presumably by antagonizing endogenous BMP signaling. In this model, the signals used to provide dorsoventral patterning in all three germ layers would be mediated by the conserved *BMP-4/chd* antagonistic system and the differences would reside in the target tissues.

When a dominant-negative FGF receptor was used to block the FGF signaling pathway, *chd* induced abundant endoderm and little neural tissue in animal cap explants. FGF signaling has been shown to be essential for mesodermal induction by activin at the blastula stage (Cornell and Kimelman, 1994; Labonne and Whitman, 1994). Whether the FGF signaling pathway is essential for neural induction may depend on the experimental conditions: Launay *et al.* (1996) reported that it is required, while Schulte-Merker and Smith (1995) reported that it is not. In our experiments, endogenous FGF signaling was required for neural induction by *chd*, but not for endodermal induction in animal cap explants.

Previous work has shown that the vegetalizing factor of Tiedemann (subsequently found to be identical to activin; Asahi *et al.*, 1979; Tiedemann *et al.*, 1992) or high levels of purified activin can lead to the formation of histologically recognizable endoderm (and mesoderm) in animal caps (Green *et al.*, 1990; Asashima *et al.*, 1991; Jones *et al.*, 1993) and to the induction of *XlHbox 8* (Gamer and Wright, 1995). Recent studies with a processed form of Vg-1 suggest an important role for this molecule in endoderm differentiation (Henry *et al.*, 1996). Taken together, these data suggest that dorsal signals from the organizer can induce pluripotent gastrula ectodermal cells to become either neural or endodermal tissues, acting in concert with various growth factors present in the embryo: FGF, BMP-4, activin/Vg-1 and, presumably, others. It is hoped that the availability of the *endodermin* pan-endodermal marker will greatly facilitate the study of endoderm differentiation in *Xenopus*.

Materials and methods

Production of chd conditioned medium and embryonic manipulations

Characterization of the Chd conditioned medium produced by baculovirus will be described elsewhere (Piccolo *et al.*, 1996). Briefly, Chd protein was overproduced using the Baculogold (Pharmingen) kit and conditioned medium bound to heparin–agarose beads (Sigma) as described (Levin *et al.*, 1995), washed twice in 1× LCMR medium (Lamb *et al.*, 1993) and grafted between two stage 9.5 animal caps in 0.3× modified Barth's solution. All animal cap results presented here were from two or more independent experiments; embryo manipulations were as described (Sasai *et al.*, 1995).

Molecular cloning of endodermin

A partial 4 kb *edd* cDNA clone (pBS-#55) was obtained in a differential screen for the genes activated in LiCl-treated dorsalized embryos but suppressed in UV-treated ventralized ones (Sasai *et al.*, 1994). A full-length cDNA clone (5.4 kb, pBS-eddL6) was obtained by rescreening 35 000 plaques of the *Xenopus* dorsal lip λZAP cDNA library with the pBS-#55 insert as a probe. The sequence of both strands was determined by the dideoxynucelotide method and analyzed by the Wisconsin GCG program.

In situ hybridization

In situ hybridizations were according to Harland's method with minor modifications (Harland, 1991; Sasai *et al.*, 1994). For the antisense *edd* digoxygenin probe, pBS-#55 was linearized with *Eco*RI and used as a template for *in vitro* transcription with T7 RNA polymerase. The *chd* probe was synthesized as described (Sasai *et al.*, 1994). It may be noted that in Figure 3D and E the hybridization stains the endoderm in some depth, which is difficult to attain in *Xenopus* whole mounts (Harland, 1991). To obtain optimal conditions to stain endoderm, we compared two commonly used alkaline phosphate substrates: BCIP/NBT and BM purple (Boehringer Mannheim). We observed that BM purple produced strong staining in superficial layers but less staining in deep embryonic layers than did BCIP/NBT. We suspect that the main difficulty in staining the *Xenopus* endoderm resides in the incubation time required for the substrate to fully penetrate the specimen. To improve this, we placed hybridized embryos in the BCIP/NTB staining solution and immediately stored them overnight at 4°C before allowing the staining reaction to proceed. In addition, we found that it helps to dilute the antibody extensively (1:20 000). Color development then requires longer times, of the order of 12–48 h, but we presume that this allows time for the substrate to penetrate the specimen more deeply.

Histological analysis

For histology, tissues or embryos were fixed in MEMFA (Harland, 1991) for 1 h, dehydrated through a series of 25, 50 and 75% methanol in phosphate-buffered saline (5 min each) and stored in 100% methanol at –20°C. For embedding, the methanol was exchanged for isopropanol twice (15 min at room temperature, 15 min at 68°C), 1:1 isopropanol:paraplast (Polysciences) for 30 min at 68°C, two changes of paraplast (1 h each at 68°C), freshly melted paraplast for 30 min and finally embedded in plastic molds (Polysciences No. 18986). After sectioning at 10 μm, slides were dried overnight at 37°C and treated (3 min each) with xylene (twice), 100 (twice), 95 and 70% ethanol and finally distilled water. After staining in Gill's hematoxylin (Fisher No. CS402-10) for 1–2 min, the slides were rinsed in tap water for 5 min, Scott's solution (Fisher No. CS410-4) for 2 min, distilled water for 2 min, 70% ethanol for 3 min and 2% eosin Y (Sigma) in 95% ethanol for 10–30 s, followed by 3 min each of 95 and 100% ethanol (twice) and xylene (twice) and mounted in Permount (Fisher).

Microinjection of synthetic mRNAs

mRNA preparation, embryo manipulations and culture conditions were as described (Sasai *et al.*, 1995). The pSP35-*chd* vector (Sasai *et al.*, 1995) was modified so that it contained a β-globin 3′ trailer and a downstream *Not*I site for linearization and was designated pSPYS-*chd*. For DNBMPR, pSP64-XTFR11-DN3 (Suzuki *et al.*, 1995) was linearized with *Eco*RI. These two constructs were more active in neural induction assays than our previous mRNAs (Sasai *et al.*, 1995). The amounts injected into each animal blastomere of the 8-cell embryo were: 75 pg for pCSKA-*chd*, 150 pg for *chd* mRNA, 75 pg for pCSKA-Bmp4, 450 pg for DNBMPR mRNA, 45 pg for *noggin* mRNA. Concentrations in the range 60–600 pg *chd* mRNA showed similar ability to induce neural and endodermal markers, while 30 pg *chd* mRNA induced less N-CAM and *edd* expression. Injected animal caps were explanted at stage 10.75 and pools of ~30 explants harvested when siblings reached stage 30. For dominant-negative FGF receptor, 450 pg of *in vitro* transcript from pSP35-*XFD* (Amaya *et al.*, 1991) was injected; other RNAs were as described (Sasai *et al.*, 1995). For FGF treatment, animal caps were explanted at stage 10.75, incubated with or without 100 ng/ml recombinant human bFGF (Promega) in 1× LCMR supplemented with 0.5% bovine serum albumin (Lamb *et al.*, 1993) for 2 h and cultured in 0.3× modified Barth solution until siblings reached stage 30.

RT–PCR

Total RNA was isolated and subjected to RT–PCR as described previously (Sasai *et al.*, 1995). The primers and conditions used were as described (Niehrs *et al.*, 1994; Sasai *et al.*, 1995; Wilson and Hemmati-Brivanlou, 1995) except for *edd* (F, 5′-TATTCTGACTCCTGAAGGTG; R, 5′-GAGAACTGCCCATGTGCCTC; 28 cycles); *XlHbox 8* (F, 5′-GCAGTCATGCTGAACCTGACAGAGAG; R, 5′-ATAGAAGGAACTTGATTGGACTGGGA; 32 cycles) and collagen type II (F, 5′-AGGCTTGGCTGGTCCTCAAGGT; R, 5′-TGTAACGCATAGGGTCGGGTCC; 24 cycles) (Su *et al.*, 1991).

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