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Ras is an essential component for notochord formation during ascidian embryogenesis

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Abstract

In ascidian embryos, inductive interactions are necessary for the fate specification of notochord cells. Previous studies have shown that notochord induction occurs at the 32-cell stage and that basic fibroblast growth factor (bFGF) has notochord-inducing activity in ascidian embryos. In vertebrate, it is known that bFGF receptors have tyrosine kinase domain and the signaling pathway is mediated by the small-GTP binding protein, Ras. To study the role of Ras in ascidian embryos, we injected dominant negative Ras (Ras^{N17}) into fertilized eggs. Ras^{N17} inhibited the formation of notochord, suggesting that the Ras signaling pathway is involved in signal transduction in the induction of notochord cells. When the presumptive-notochord (A6.2) blastomere was co-isolated with the inducer (A6.1) blastomere and then Ras^{N17} was injected into the A6.2 blastomere, notochord differentiation was suppressed. The presumptive-notochord blastomeres injected with Ras^{N17} were treated with bFGF. Many of them failed to develop notochord-specific features. Next, we examined the effect of injecting constitutively active Ras (Ras^{V12}) into the A6.2 blastomeres. However, microinjection of Ras^{V12} into these cells did not bypass notochord induction. These results suggest that the Ras signaling pathway is essential for the formation of notochord and that another signaling pathway also must be activated simultaneously in notochord formation during ascidian embryogenesis. © 1997 Elsevier Science Ireland Ltd.

Keywords: Ascidian embryogenesis; Notochord induction; bFGF; Ras signaling

1. Introduction

An ascidian larva contains 40 notochord cells located along the midline of the tail aligned longitudinally in a single row. Among these 40 notochord cells, 32 cells situated in the anterior and middle regions of the tail arise from eight precursor blastomeres of the A-line in the 110-cell embryo, while the other eight notochord cells situated in the posterior tail region are derived from the two precursor blastomeres of the B-line in the 110-cell embryo. The two lineages of the A-line and the B-line precursor blastomeres contribute to the formation of notochord cells of the tadpole larva (Nishida, 1987).

Notochord differentiation of the A-line cells depends on cellular interactions between the presumptive-notochord blastomeres and the inducer blastomeres at the 32-cell stage (Nakatani and Nishida, 1994). Blastomeres of the vegetal hemisphere that include the presumptive-endoderm blastomeres and the presumptive-notochord blastomeres themselves are inducers of notochord differentiation. When the presumptive-notochord (A6.2 or A6.4) blastomeres at the early 32-cell stage are manually isolated and cultured alone, they do not differentiate into notochord (Fig. 1A). Co-isolation or recombination of these presumptive-notochord blastomeres with the inducer blastomeres results in notochord-specific features (Fig. 1B,C). These results suggest that in the A-line precursors, notochord cells are formed in response to inductive influences from the presumptive-endoderm (A6.1 or A6.3) blastomeres and the presumptive-notochord blastomeres themselves. In contrast, the determinative mechanism of the B-line notochord cells remains to be clarified, because any combinations of the B-line blastomeres do not show the differentiation of notochord.

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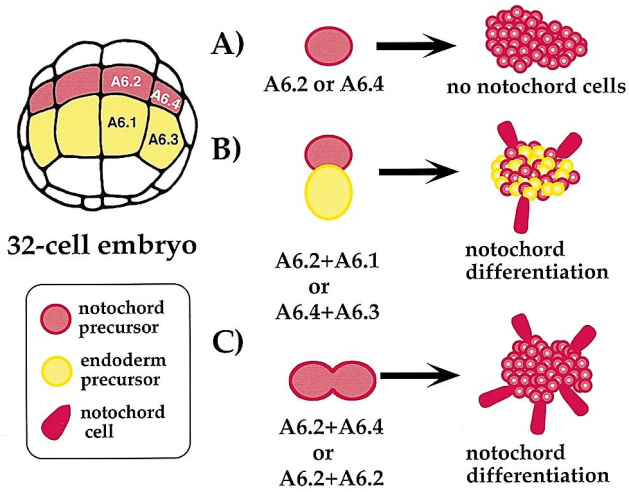

Fig. 1. Experimental design of notochord induction experiments in ascidian embryos. (A) The presumptive-notochord (A6.2 and A6.4) blastomeres are manually isolated from the 32-cell stage embryo. They are then allowed to develop as partial embryos. No notochord differentiation is observed. (B,C) Co-isolation or recombination experiments. A notochord precursor blastomere (A6.2 and A6.4) is isolated together (co-isolation) or isolated separately and then recombined (recombination) with an inducer blastomere, that is either an endoderm precursor (A6.1 and A6.3) blastomere (B) or an another notochord precursor blastomere (C). In both cases a significant proportion of the partial embryos develops notochord-specific features (Nakatani and Nishida, 1994).

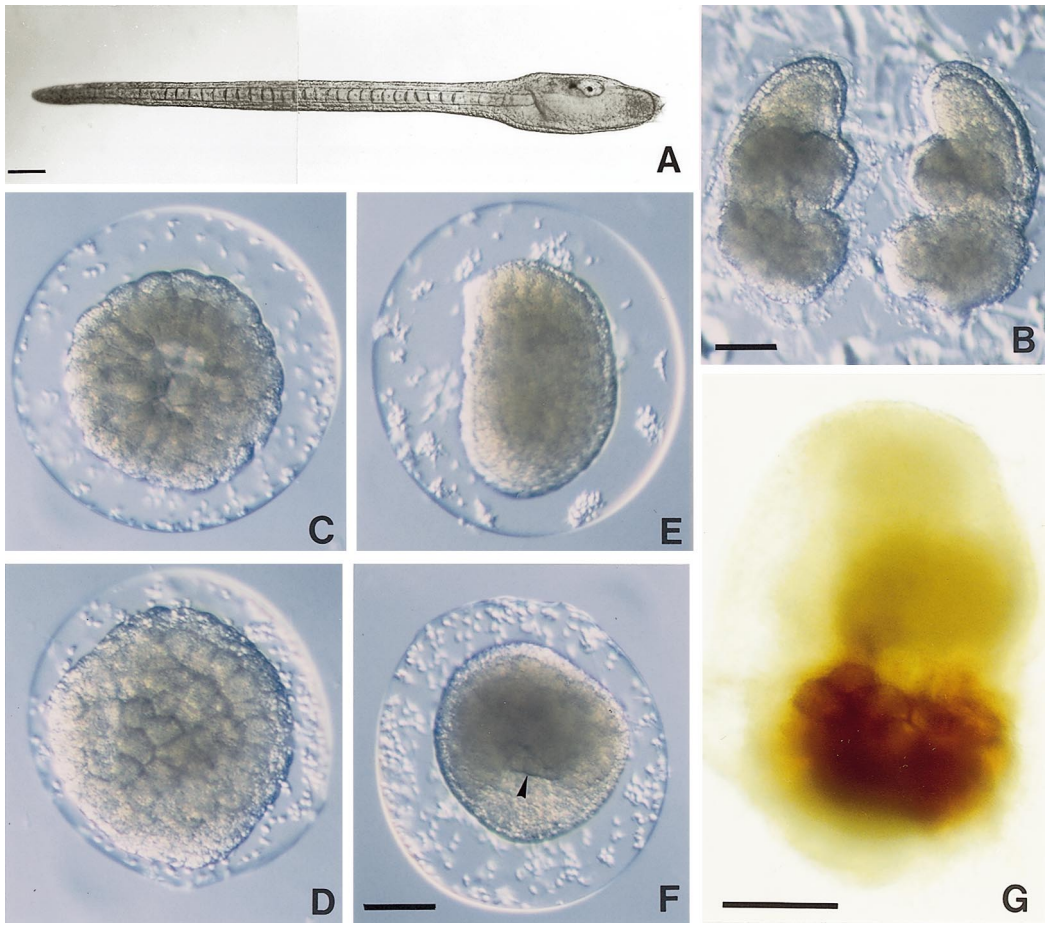

Fig. 2. Morphological comparisons between normal and Ras^{N17}-injected embryos. (A,C,E) Embryos without injection. (B,D,F,G) Embryos that were injected with 0.88 mg/ml Ras^{N17} just after fertilization, showing abnormal morphology. (A) A normal ascidian larva after hatching. (B) Two Ras^{N17}-injected embryos at the tailbud stage. Putative anterior is up and putative posterior is down. In the posterior region the tail was shortened. (C,D) Early gastrula stage viewed from the vegetal pole. Vegetal cells are invaginating in (C) but not in (D). (E,F) Late neurula stage. Dorsal is to the left in (E). Blastopore (arrow) is still open in (F). (G) AchE, which is a marker of muscle cells, is expressed in the putative posterior region. Scale bars, 100 μm.

Recent work for testing notochord-inducing activity demonstrated that bFGF, but not activin, can induce notochord differentiation in the presumptive-notochord blastomeres of the A-line isolated from the early 32-cell embryos (Nakatani et al., 1996). The biological activity of bFGF is mediated through cell surface receptors that have an associated tyrosine kinase activity (Lee et al., 1989). Much progress has been made toward understanding how tyrosine kinase receptors transmit signals and it is suggested that the Ras signaling pathway is activated by these receptors (for reviews see Satoh et al., 1992; Lowy and Willumsen, 1993; Pawson, 1995). Since much less is known about the signal transduction pathway in ascidian embryos, it is not yet clear how cells integrate the extracellular signals. To explore the intracellular signaling pathways in ascidian embryos, we microinjected mutant Ras protein into ascidian embryos. In the present study, we show that the injection of a dominant negative mutant of Ras, Ras^{N17}, into A6.2 notochord progenitor cells suppressed notochord formation. It is therefore suggested that Ras is an important downstream component during inductive interactions required for notochord formation in ascidian embryos.

2. Results

2.1. Dominant negative mutant of Ras inhibits notochord formation in ascidian embryos

To determine whether Ras plays a role during ascidian embryogenesis, we injected dominant negative Ras (Ras^{N17}) into fertilized eggs. The concentration of Ras^{N17} injected was 0.88 mg/ml. Eggs injected with Ras^{N17} developed into morphologically abnormal larvae (Fig. 2B). Most of the embryos which were injected with Ras^{N17} had no elongated tail (95%) surrounded by epidermis. In the Ras^{N17}-injected embryos, elongated notochords were not observed. A normal ascidian larva has a brain (sensory vesicle) and two kinds of sensory pigment cells, ocellus and otolith, in its brain (Fig. 2A). The Ras^{N17}-injected embryos did not develop pigment cells, or a morphologically identifiable brain.

When the development of injected embryos was observed, no differences between normal and injected embryos were observed until the 110-cell stage. In normal ascidian embryogenesis, gastrulation starts after the 110-cell stage (approximately 9 h after fertilization at 13°C) (Fig. 2C). Injected embryos exhibited a delay in the start of gastrulation as compared to normal embryos (Fig. 2C–F). At the stage that corresponds normally to early gastrula, the Ras^{N17}-injected embryos did not start to invaginate and the vegetal region of these embryos remained flattened (Fig. 2D). After a delay of 2 h, injected embryos started gastrulation. At the neurula stage of normal embryos (about 15 h after fertilization at 13°C) (Fig. 2E), the Ras^{N17}-injected embryos had the blastopore still opening (Fig. 2F). No evidence of neural tube formation was observed. At the stage corresponding normally to the tailbud, all the injected embryos displayed a truncated axis posteriorly and short tailed larvae developed, while the tail was elongated in normally developed embryos at this stage (Fig. 2B). This phenotype was also observed in *Xenopus* embryos when dominant negative FGF receptor (Amaya et al., 1991), dominant negative ras (Whitman and Melton, 1992), dominant negative raf (MacNicol et al., 1993) and mitogen-activated protein kinase (MAPK) phosphatase (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995) were microinjected (see Section 3).

Differentiation of various tissues was examined by monitoring the expression of molecular markers (Table 1). The Epi-2 antigen, which is a marker of differentiated epidermis, was expressed in the surrounding epithelium of these abnormal larvae. Alkaline phosphatase (AP) activity, a marker of endoderm, was detected in the putative anterior region. Muscle cells, which express acetylchorinesterase (AchE), pile up in the putative posterior region, which is likely to be a shortened tail (Fig. 2G). Thus, Ras^{N17}-injected embryos were viable and developed epidermis, endoderm and muscle cell markers. It is plausible that shortening of the tail is a consequence of the absence of notochord differentiation. Notochord differentiation was repressed, as judged by expression of the Not-1 antigen. At the tailbud stage normal embryos expressed the Not-1 antigen in their tail region (Fig. 3A), while the injected embryos did not express the Not-1 antigen (9%) (Fig. 3B). To further confirm the absence of notochord formation in the Ras^{N17}-injected

Table 1
Expression of tissue-specific features in Ras^{N17}-injected embryos

Injected materials	No. of partial embryos with tissue-specific features/no. of embryos examined (%, percentage of embryos examined)				
	Epi-2	AP	AchE	Not-1	Not-1[a] (110-cell)
No injection	25/25 (100)	22/22 (100)	52/52 (100)	64/64 (100)	38/38 (100)
Ras^{N17}	26/26 (100)	24/24 (100)	23/23 (100)	3/35 (9)	6/18 (33)
Injection buffer	22/22 (100)	25/25 (100)	48/48 (100)	43/43 (100)	19/19 (100)

Fertilized eggs were injected with Ras^{N17}. As a control, injection buffer was microinjected into fertilized eggs. Then eggs were cultured in MFSW. Differentiation of tissue-specific features was examined by monitoring the expression of various molecular markers: Epi-2, epidermis; AP, endoderm; AchE, muscle; Not-1, notochord.

[a]Cell divisions of embryos were continuously inhibited after the 110-cell stage.

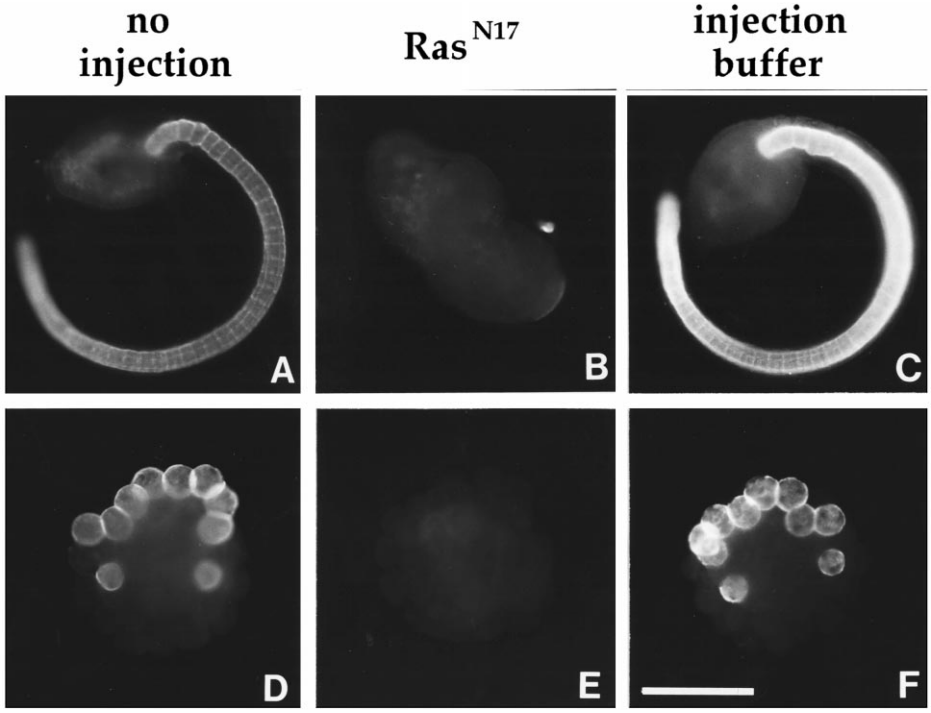

Fig. 3. Expression of the Not-1 antigen. (A,D) No injection. (B,E) Injection of Ras^{N17}. (C,F) Injection of the injection buffer. (A–C) Expression of the Not-1 antigen at the late tailbud stage. Staining of the Not-1 antigen can be observed in the surfaces of notochord cells (A,C), while no expression of the Not-1 antigen was detected in the Ras^{N17}-injected embryos (B). (D–F) Cell divisions of embryos were continuously inhibited after the 110-cell stage. (D,F) Expression of the Not-1 antigen was detected by immunostaining. There is a row of eight Not-1 positive cells, which are probably A-line notochord precursor cells, and two additional positive cells, which are derived from the B-line precursor cells. (E) No positive cells were detected in Ras^{N17}-injected embryos. Scale bar, 100 μm.

embryos, cleavage was arrested by treatment with cytochalasin B after the 110-cell stage and notochord differentiation was ascertained by monitoring the expression of the Not-1 antigen. In normal arrested embryos, notochord precursor cells expressed the Not-1 antigen (100%) (Fig. 3D). The maximum number and position of the Not-1 expressing cells were consistent with those of the notochord-lineage cells at the 110-cell stage. This result coincided with previous results reported by Nishikata and Satoh (1990). In the Ras^{N17}-injected embryos, the expression of the Not-1 antigen was abolished in 67% of specimens (Fig. 3E). These observations suggest that Ras^{N17} can sometimes suppress notochord cell differentiation, but do not affect the differentiation of epidermis, endoderm and muscle cells.

In control experiments, eggs were injected with the injection buffer (buffer A plus 50% glycerol). No morphological abnormalities were detected in these larvae. These larvae had a normal number of notochord cells (40 cells) and expressed the Not-1 antigen at the tailbud stage (Fig. 3C). They also expressed the Not-1 antigen when cleavage arrested (Fig. 3F).

2.2. Ras^{N17} blocks the endogenous signals in notochord formation

We further analyzed the involvement of Ras in notochord formation at the single cell level by the experiments with co-isolation of blastomeres. A previous study has demonstrated that co-isolation of the presumptive-notochord (A6.2) blastomere together with the inducer blastomere, that is the presumptive-endoderm (A6.1) blastomere, at the early 32-cell stage results in expression of the notochord-specific features, as notochord induction occurs between two blastomeres within co-isolates (Fig. 1B) (Nakatani and Nishida, 1994). At the 32-cell stage, the A6.2 blastomere and the A6.1 blastomere were similarly co-isolated together and then Ras^{N17} was microinjected into the presumptive-notochord (A6.2) blastomeres. The concentration of Ras^{N17} injected was 0.88 mg/ml. These injected co-isolates were cultured in MFSW as partial embryos. Notochord differentiation was detected morphologically as well as by monitoring the expression of the Not-1 antigen. The results are summarized in Table 2.

As the morphological criteria, notochord cells are elongated and have a vacuole on one side of each cell (Fig. 4A, arrow). They also express notochord-specific antigen. Co-isolates of an A6.2 blastomere with an adjacent A6.1 blastomere differentiated into notochord cells (95–100%) (Fig. 4A,E). When Ras^{N17} was microinjected into an A6.2 blastomere that was co-isolated with an A6.1 blastomere, the frequency of differentiation of notochord was dramatically reduced (5–13%) (Fig. 4B,F). As control experiments, the injection buffer or wild-type Ras was injected into an A6.2 blastomere of co-isolates. The concentration of wild-type

Table 2
Expression of notochord-specific features in co-isolated blastomeres

Injected materials	No. of partial embryos with notochord-specific features/no. of embryos examined (%)	
	Morphology	Not-1
No injection	36/38 (95)	21/21 (100)
Ras^{N17}	1/19 (5)	2/15 (13)
Injection buffer	19/22 (86)	16/16 (100)
Wild-type Ras	16/18 (89)	14/16 (88)

The presumptive-notochord (A6.2) blastomeres and the presumptive-endoderm (A6.1) blastomeres were co-isolated from the 32-cell embryos and Ras^{N17} was microinjected into the A6.2 blastomeres. Then these co-isolated blastomeres were cultured in MFSW. Expression of notochord-specific features were examined morphologically and immunohistochemically (Not-1). As control experiments, injection buffer and wild-type Ras were injected.

Ras injected was 0.94 mg/ml. Both of these control co-isolates developed notochord cells, recognizable by their morphology and by immunodetection (Fig. 4C,D,G,H). These results suggest that endogenous signaling that occurs in the presumptive-notochord blastomere after receiving inductive influence from the inducer were inhibited by Ras^{N17}.

2.3. Notochord induction by bFGF was repressed by Ras^{N17}

Treatment with bFGF of isolated presumptive-notochord blastomeres from the early 32-cell embryos results in notochord differentiation. The sensitive period to bFGF is restricted to the 32-cell stage (Nakatani et al., 1996). In the present study, the A6.2 blastomeres were manually isolated from the early 32-cell embryos (Fig. 1A) and then Ras^{N17} was injected into the blastomeres. The injected blastomeres were cultured in MFSW that contained 0.1% BSA plus 2 ng/ml bFGF. The concentration of Ras^{N17} injected was 0.88 mg/ml. Notochord formation was judged by both the morphology and the expression of the Not-1 antigen. The results were summarized in Table 3 (series (a)). Treatment with 2 ng/ml bFGF induced the differentiation of notochord in non-injected A6.2 partial embryos (100%) (Fig. 5A,D). Notochord differentiation was observed when an A6.2 blastomere was microinjected with the injection buffer and treated with 2 ng/ml bFGF (100%) (Fig. 5B,E). By contrast, the frequency of notochord formation was reduced in the A6.2 isolates injected with Ras^{N17} (11–51%) (Fig. 5C,F). These results suggest that Ras^{N17} is capable of suppressing the bFGF-mediated notochord.

In the next series of experiments, we injected the constitutively active form of Ras (Ras^{V12}) into the presumptive-notochord (A6.2) blastomeres isolated at the early 32-cell stage. Ras^{V12}, whose ability to bind with Raf has been confirmed in vitro, was used for microinjection at a concentration of 0.90 mg/ml. As Ras^{N17} can prevent notochord formation, Ras^{V12} might bypass the reception of signal for notochord induction even if no inducing signal exists. The results are summarized in Table 3 (series (b)). Previous studies have demonstrated that the A6.2 isolates without inducing signals do not show any features of notochord when they are cultured in MFSW or MFSW containing only 0.1% BSA (Fig. 1A) (Nakatani and Nishida, 1994; Nakatani et al., 1996) and this was reconfirmed (Fig. 5G,J). Ras^{V12} was injected into the isolated A6.2 blastomeres without inducers and then injected A6.2 blastomeres were cultured in MFSW. Most of the A6.2 isolates injected with Ras^{V12} did not develop notochord-specific features (Fig. 5H,I,K,L). In control experiments, wild-type Ras was injected into the A6.2 isolates and cultured in MFSW. These injected isolates did not differentiate into notochord.

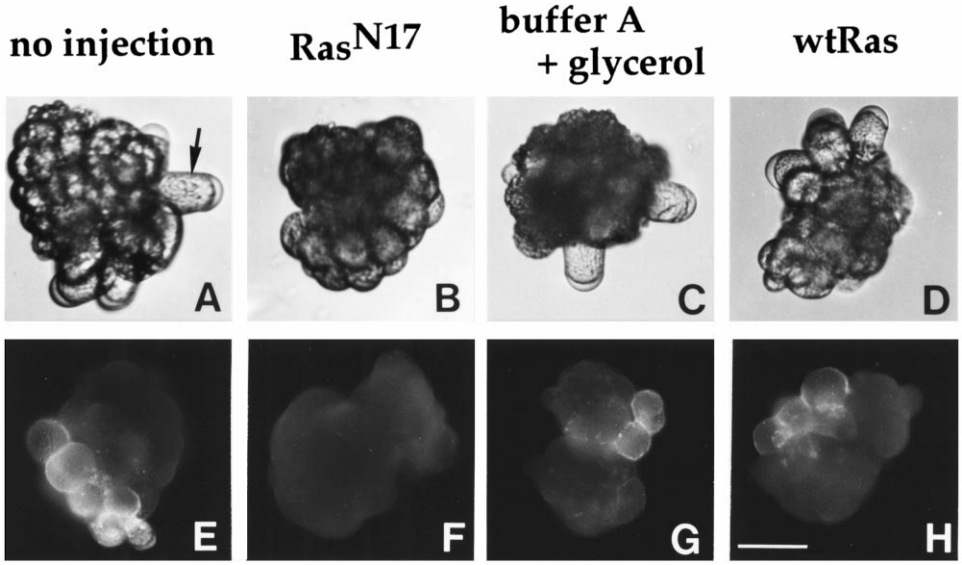

Fig. 4. Photomicrographs of partial embryos obtained in co-isolation experiments. An A6.2 blastomere and an A6.1 blastomere were co-isolated manually from the 32-cell embryos. Uninjected cells are shown in (A,E). The A6.2 blastomeres were injected with Ras^{N17} (B,F), the injection buffer (C,G) and wild-type Ras (D,H). (A–D) The morphology of partial embryos is shown. Notochord cells have a vacuole and an elongated profile, as indicated by the arrow. (E–H) Expression of the Not-1 antigen, as visualized by immunofluorescence. Scale bar, 50 μm.

Table 3
Expression of notochord-specific features in the A6.2 blastomeres

Injected materials	Treatment	No. of partial embryos with notochord-specific features/no. of embryos examined (%)	
		Morphology	Not-1
Series (a)			
No injection	2 ng/ml bFGF	33/33 (100)	20/20 (100)
Injection buffer	2 ng/ml bFGF	24/24 (100)	17/17 (100)
Ras^{N17}	2 ng/ml bFGF	20/39 (51)	2/19 (11)
Series (b)			
No injection	BSA-SW	0/33 (0)	0/21 (0)
Wild-type Ras	MFSW	1/18 (6)	0/20 (0)
Ras^{V12}	MFSW	0/17 (0)	0/22 (0)

In the first series of experiments (a), the presumptive-notochord (A6.2) blastomeres were isolated at the 32-cell stage, injected with dominant negative Ras^{N17} and then cultured in MFSW plus 0.1% BSA with 2 ng/ml bFGF. As a control, injection buffer was microinjected into the A6.2 blastomeres.
In the second series of experiments (b), the A6.2 blastomeres isolated at the 32-cell stage were microinjected with constitutively active Ras^{V12} and then cultured in MFSW or plus only 0.1% BSA (BSA-SW). As a control, wild-type Ras was injected. Notochord differentiation was monitored in terms of morphology and the expression of the Not-1 antigen.

The present study shows that Ras^{N17} can block the responsiveness to both the endogenous notochord-inducing signal(s) and treatment with bFGF, while Ras^{V12} was insufficient to induce the differentiation of notochord.

3. Discussion

The results described in the present report suggest that a Ras signaling pathway functions during early ascidian development and that Ras is an essential component for induction of notochord. Microinjection of dominant negative Ras^{N17} into fertilized eggs eliminated notochord formation. Ras^{N17} prevented notochord differentiation in response to both endogenous signals from the inducer blastomeres and an exogenous signal, human recombinant bFGF. These results indicate that Ras activation is required for notochord formation.

Many intercellular signals are transmitted via membrane-bound receptor tyrosine kinases. Tyrosine kinase signaling has been well studied and is the subject of intense study (for a review see Lemmon and Schlessinger, 1994). After ligand binding, oligomerization of the receptors occurs, which results in an increase of the intrinsic tyrosine kinase activity and phosphorylation of several cytoplasmic tyrosine residues within the receptor. Activation of the receptor leads to the activation of Ras, which in turn activates raf-1 and MAPK. During early *Xenopus* development, these intracellular molecules activated by tyrosine kinase receptors have been shown to be important for the signal transduction in mesoderm induction (Whitman and Melton, 1992; MacNicol et al., 1993; Graves et al., 1994; Hartley et al., 1994; LaBonne and Whitman, 1994; Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995). The receptors for bFGF encode protein tyrosine kinases. Exogenous bFGF can activate these signaling molecules for mesoderm induction in *Xenopus* embryos (LaBonne and Whitman, 1994). Ras^{N17} inhibits mesoderm induction by both bFGF and the endogenous inducing signals, suggesting the requirement for Ras signaling pathway during *Xenopus* mesoderm induction. Moreover, constitutively active Ras promotes the expression of some mesodermal molecular markers and the formation of mesodermal tissues, indicating that activation of

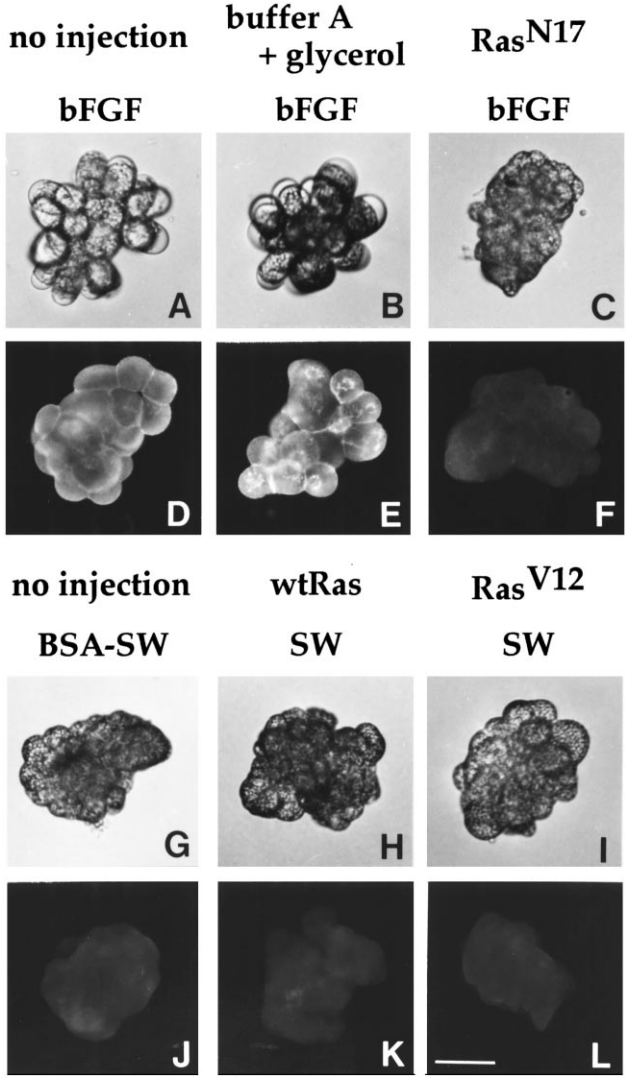

Fig. 5. Photomicrographs of A6.2 partial embryos. In the first series, shown in (A–F), A6.2 isolates developed in MFSW plus 0.1% BSA that contained bFGF at a concentration of 2 ng/ml. In the next series shown in (G–L), A6.2 isolates were cultured in MFSW without bFGF. Partial embryos that developed without microinjection are shown in (A,D,G,J). Partial embryos injected with the injection buffer are shown in (B,E). Ras^{N17} injected embryos are shown in (C,F), wild-type Ras injected embryos are shown in (H,K) and Ras^{V12} injected embryos are shown in (I,L). (A–C,G–I) The morphology of partial embryos. (D–F,J–L) The expressions of the Not-1 antigen. Scale bar, 50 μm.

Ras alone is sufficient to bypass mesoderm inducing signals in *Xenopus* (Whitman and Melton, 1992; LaBonne and Whitman, 1994).

In ascidian embryos, Ras signaling pathway is essential for the formation of notochord. However, microinjection of constitutively active Ras did not promote notochord formation. Thus, the mechanism of bFGF/Ras-mediated notochord induction in ascidian embryos may differ in some respects from the mechanism of bFGF/Ras-mediated mesoderm induction in *Xenopus* embryos. It is known that some transmembrane tyrosine kinase receptors, which include the epidermal growth factor (EGF), the colony stimulating factor 1 (CSF-1) and the platelet-derived growth factor (PDGF) receptors, can activate the Janus protein-tyrosine kinases (JAKs) and the activated JAKs phosphorylate the signal transducers and activators of transcription (STATs). This JAK/STAT pathway is independently activated by the Ras pathway (for review see Ihle, 1996). Thus, there are at least two independent pathways downstream of the tyrosine kinase receptors. Although based on negative results, activation of another signaling pathway other than the Ras pathway downstream of putative bFGF receptor may be necessary for the formation of notochord in ascidian embryos. One possibility is that the JAK/STAT pathway may be involved in ascidian notochord formation.

Injection of Ras^{N17} into fertilized eggs caused the elimination of differentiated notochord and also inhibited the formation of brain and sensory pigment cells in the larvae. In ascidian embryos, inductive interactions are known to be necessary for formation of brain and sensory pigment cells (Rose, 1939; Reverberi and Minganti, 1946; Okado and Takahashi, 1988; Nishida and Satoh, 1989). Although we did not further analyze on these phenomena, it is plausible that Ras signaling pathway is also directly or indirectly involved in the formation of these neural tissues (brain and two sensory pigment cells).

During embryogenesis, the Ras^{N17}-induced defects were first observed morphologically at the gastrula stage. In the Ras^{N17}-injected embryos, the gastrulation was delayed and the blastopore failed to close. This phenotype is similar to that observed when dominant negative FGF receptor (Amaya et al., 1991), dominant negative ras (Whitman and Melton, 1992), dominant negative raf (MacNicol et al., 1993) and MAPK phosphatase (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995) were injected into early *Xenopus* embryos. In *Xenopus* embryos, when dominant negative FGF receptor, dominant negative ras, dominant negative raf and MAPK phosphatase were injected, the injected embryos failed to complete gastrulation. Early movements of gastrulation occurred normally and on time, and at the late gastrula stage normal closure of the blastopore was delayed. At the tailbud stage, head structures were observed, but the injected embryos showed severe posterior truncation, resulting in defects of the tail structure. Thus, Ras-dependent events may be involved in gastrulation movements both in *Xenopus* and ascidian embryos.

Microinjection of Ras^{N17} inhibited responsiveness both to the endogenous inducer and to human recombinant bFGF, which resulted in the absence of notochord differentiation in the A6.2 blastomeres. When Ras^{N17} was injected into an A6.2 blastomere that was co-isolated with an A6.1 blastomere, the frequency of notochord differentiation was dramatically decreased (Table 2). By contrast, the suppression of notochord induction was slightly weak when the A6.2 blastomeres that were injected with Ras^{N17} were treated with 2 ng/ml bFGF (Table 3). The concentration of injected Ras^{N17} may be too low to completely suppress the signaling activated by the rather high concentration of bFGF.

4. Experimental procedures

4.1. Embryos

Naturally spawned eggs of *Halocynthia roretzi* were cross-fertilized in vitro. Fertilized eggs were reared in Millipore-filtered (pore size, 0.45 μm) seawater that contained 50 μg/ml streptomycin sulfate and 50 μg/ml kanamycin sulfate (MFSW) at 13°C, at which temperature tadpole larvae hatched about 35 h after fertilization.

4.2. Microinjection of human Ras into ascidian embryos

The wild-type Ras, dominant negative Ras (Ras^{N17}) and constitutively active Ras (Ras^{V12}) proteins, which were expressed in *Escherichia coli* and purified according to standard procedures (Miura et al., 1986) were kindly provided by Dr. H. Koide and Dr. Y. Kaziro (Tokyo Institute of Technology, Japan). All the Ras proteins were solubilized in injection buffer, which consists of buffer A (50 mM HEPES NaOH (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF) plus 50% glycerol. Before microinjection, we prepared a 1:1 mixture (v/v) of Ras solution and 1 mg/ml fast green in water. The final concentration of Ras protein to be injected was approximately 1 mg/ml.

Fertilized eggs were used for microinjection with Ras proteins. After fertilization, eggs were treated with 0.05% actinase E (Kaken, Tokyo, Japan) in seawater for 3 min. Follicle cells outside the vitelline membrane were removed during the incubation and after thorough washing eggs were allowed to adhere to a coverslip in a plastic dish filled with MFSW. Microinjection by pressure was performed through the vitelline membrane without the follicle cells. Microinjections were performed using injection pipettes that were held by micromanipulators (Model MN-151, Narishige, Tokyo, Japan) under a stereomicroscope (SZH-10, Olympus, Japan). The injected volume of Ras solution was approximately 10% of the total volume of eggs and blastomeres.

The procedure of microinjection of Ras proteins into blastomeres was as follows. Fertilized eggs were manually dechorionated with sharpened tungsten needles and reared in 0.9% agar-coated plastic dishes that contained MFSW. At the 32-cell stage, identified blastomeres were isolated from embryos with a fine glass needle under a stereomicroscope. Then isolated blastomeres were injected with the solution containing Ras proteins. They were cultured separately as partial embryos in MFSW or in MFSW that contained 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) with 2 ng/ml bFGF. Recombinant human bFGF was purchased from Amersham (Buckinghamshire, UK).

4.3. Cleavage arrest of embryos

The 110-cell embryos were incubated in MFSW that contained 2.5 μg/ml cytochalasin B (Aldrich Chemical, Milwaukee, WI, USA) until control embryos reached the tailbud stage and then they were immunostained with a notochord-specific monoclonal antibody.

4.4. Markers for tissue differentiation

The monoclonal antibody 5F1D5 recognizes a notochord-specific antigen, Not-1 (Nishikata and Satoh, 1990). At the tailbud stage (20 h), this antibody is strictly specific to staining notochord cells. Therefore, specimens were fixed and immunostained at the tailbud stage (Nakatani and Nishida, 1994). The monoclonal antibody, Epi-2, recognizes the larval epidermis cells and the larval tunic, which is secreted by epidermal cells (Nishikata et al., 1987). Indirect immunohistochemical staining was carried out by the standard methods using fluorescein isothiocyanate (FITC)-conjugated second antibodies. These tissue-specific antibodies were kindly provided by Dr. T. Nishikata (Konan University, Japan).

Differentiation of endoderm was monitored by histochemical detection of alkaline phosphatase (AP) activity using the method of Whittaker and Meedel (1989) with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrate. The formation of muscle was monitored by histochemistry for acetylchorinesterase (AchE). Histochemical staining of AchE activity was carried out as described by Karnovsky and Roots (1964) with acetylcholine iodide as the substrate.

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References

Amaya, E., Musci, T.J., Kirschner, M.W., 1991. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. Cell 66, 257–270.

Gotoh, Y., Masuyama, N., Suzuki, A., Ueno, N., Nishida, E., 1995. Involvement of the MAP kinase cascade in *Xenopus* mesoderm induction. EMBO J. 14, 2491–2498.

Graves, L.M., Northrop, J.L., Potts, B.C., Krebs, E.G., Kimelman, D., 1994. Fibroblast growth factor, but not activin, is a potent activator of mitogen-activated protein kinase in *Xenopus* explants. Proc. Natl. Acad. Sci. USA 91, 1662–1666.

Hartley, R.S., Lewellyn, L., Maller, J.L., 1994. MAP kinase is activated during mesoderm induction in *Xenopus laevis*. Dev. Biol. 163, 521–524.

Ihle, J.N., 1996. STATs: signal transducers and activators of transcription. Cell 84, 331–334.

Karnovsky, M.J., Roots, L., 1964. A 'direct-coloring' thiocholine method for cholinesterase. J. Histochem. Cytochem. 12, 219–221.

LaBonne, C., Whitman, M., 1994. Mesoderm induction by activin requires FGF-mediated intracellular signals. Development 120, 463–472.

LaBonne, C., Burke, B., Whitman, M., 1995. Role of MAP kinase in mesoderm induction and axial patterning during *Xenopus* development. Development 121, 1475–1486.

Lee, P.L., Johnson, D.E., Cousens, L.S., Fried, V.A., Williams, L.T., 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science 245, 57–60.

Lemmon, M.A., Schlessinger, J., 1994. Regulation of signal transduction and signal diversity by receptor oligomerization. Trends Biochem. Sci. 19, 459–463.

Lowy, D.R., Willumsen, B.M., 1993. Function and regulation of ras. Annu. Rev. Biochem. 62, 851–891.

MacNicol, A.M., Muslin, A.J., Williams, L.T., 1993. Raf-1 kinase is essential for early *Xenopus* development and mediates the induction of mesoderm by FGF. Cell 73, 571–583.

Miura, K., Inoue, Y., Nakamori, H., Iwai, S., Ohtsuka, E., Ikehara, M., Noguchi, S., Nishimura, S., 1986. Synthesis and expression of a synthetic gene for the activated human c-Ha-*ras* protein. Jpn. J. Cancer Res. (Gann) 77, 45–51.

Nakatani, Y., Nishida, H., 1994. Induction of notochord during ascidian embryogenesis. Dev. Biol. 166, 289–299.

Nakatani, Y., Yasuo, H., Satoh, N., Nishida, H., 1996. Basic fibroblast growth factor induces notochord formation and the expression of *As-T*, a *Brachyury* homolog, during ascidian embryogenesis. Development 122, 2023–2031.

Nishida, H., 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. Dev. Biol. 121, 526–541.

Nishida, H., Satoh, N., 1989. Determination and regulation in the pigment cell lineage of the ascidian embryo. Dev. Biol. 132, 355–367.

Nishikata, T., Satoh, N., 1990. Specification of notochord cells in the

ascidian embryo analysed with a specific monoclonal antibody. Cell Diff. Dev. 30, 43–53.

Nishikata, T., Mita-Miyazawa, I., Deno, T., Satoh, N., 1987. Expression of epidermis-specific antigens during embryogenesis of the ascidian *Halocynthia roretzi*. Dev. Biol. 121, 408–416.

Okado, H., Takahashi, K., 1988. A simple 'neural induction' model with two interacting cleavage-arrested ascidian blastomeres. Proc. Natl. Acad. Sci. USA 85, 6197–6201.

Pawson, T., 1995. Protein modules and signaling networks. Nature 373, 573–580.

Reverberi, G., Minganti, A., 1946. Fenomeni di evocazione nello sviluppo dell'uovo di Ascidie. Pubbl. Staz. Zool. Napoli 20, 199–252.

Rose, S.M., 1939. Embryonic induction in the ascidia. Biol. Bull. Mar. Exp. 3, 296–336.

Satoh, T., Nakafuku, M., Kaziro, Y., 1992. Function of ras as a molecular switch in signal transduction. J. Biol. Chem. 267, 24149–24152.

Umbhauer, M., Marshall, C.J., Mason, C.S., Old, R.W., Smith, J.C., 1995. Mesoderm induction in *Xenopus* caused by activation of MAP kinase. Nature 376, 58–62.

Whitman, M., Melton, D.A., 1992. Involvement of $p21^{ras}$ in *Xenopus* mesoderm induction. Nature 357, 252–254.

Whittaker, J.R., Meedel, T.H., 1989. Two histospecific enzyme expressions in the same cleavage-arrested one-celled ascidian embryos. J. Exp. Zool. 250, 168–175.