JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates

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Received July 12, 2001; revised and accepted November 15, 2001

Recent genetic studies in *Drosophila* identified a novel non-canonical Wnt pathway, the planar cell polarity (PCP) pathway, that signals via JNK to control epithelial cell polarity in *Drosophila*. Most recently, a pathway regulating convergent extension movements during gastrulation in vertebrate embryos has been shown to be a vertebrate equivalent of the PCP pathway. However, it is not known whether the JNK pathway functions in this non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. In addition, it is not known whether JNK is in fact activated by Wnt stimulation. Here we show that Wnt5a is capable of activating JNK in cultured cells, and present evidence that the JNK pathway mediates the action of Wnt5a to regulate convergent extension movements in *Xenopus*. Our results thus demonstrate that the non-canonical Wnt/JNK pathway is conserved in both vertebrate and invertebrate and define that JNK has an activity to regulate morphogenetic cell movements.

INTRODUCTION

Studies in *Xenopus* suggested that Wnt ligands can be classified into at least two subfamilies (Moon *et al.*, 1993b); one is the Wnt1 class that includes Wnt8 and signals mainly through the canonical Wnt/ β -catenin pathway to control cell growth and cell fate specification (Cadigan and Nusse, 1997; Miller *et al.*, 1999; Sokol, 1999). The other is the Wnt5a class that includes Wnt11 and signals mainly through the planar cell polarity (PCP) pathway to regulate convergent extension movements in developing embryos (Moon *et al.*, 1993a; Ungar *et al.*, 1995; Torres *et al.*, 1996; Heisenberg *et al.*, 2000; Tada and Smith,

2000; Wallingford *et al.*, 2000). In fact, in *Xenopus* embryos expressing dominant-negative Wnt11 and in zebrafish mutants lacking a functional *Wnt11* gene, convergent extension is strongly inhibited (Heisenberg *et al.*, 2000; Tada and Smith, 2000). It has been shown that Dishevelled, an intracellular protein, mediates not only the canonical Wnt/ β -catenin pathway but also the PCP pathway in both *Drosophila* and vertebrates (Sokol, 1996; Axelrod *et al.*, 1998; Boutros *et al.*, 1998; Heisenberg *et al.*, 2000; Tada and Smith, 2000; Wallingford *et al.*, 2000), and that the MKK7/JNK pathway functions downstream of Dishevelled to mediate the PCP pathway in *Drosophila* (Boutros *et al.*, 1998). In vertebrates, however, signaling pathways downstream of Dishevelled in the PCP pathway during convergent extension movements have been undefined. Moreover, in both *Drosophila* and vertebrates, activation of JNK during Wnt stimulation has not been demonstrated, although overexpression of Dishevelled protein in cultured cells is shown to activate JNK and its direct activator MKK7 (Boutros *et al.*, 1998; Li *et al.*, 1999; Moriguchi *et al.*, 1999; Davis, 2000). Here we demonstrate that JNK is in fact activated by Wnt5a and that the JNK pathway mediates the action of Wnt5a to regulate convergent extension movements in *Xenopus*.

RESULTS

JNK is activated by Wnt5a

We first assayed whether Wnt stimulation is capable of activating JNK in cultured cells. We tested two *Xenopus* Wnt ligands, *Xenopus* Wnt5a and *Xenopus* Wnt8, for their activity to

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activate members of the MAP kinase family in co-expression experiments. Among these members of the MAP kinase family examined, only JNK, but not ERK or p38, was significantly activated by Wnt5a (Figure 1A and B). Wnt8 did not activate significantly any of these members of the MAP kinase family (Figure 1A and B). In these experiments, it was confirmed that appropriate activators could activate all these transfected kinases. To further confirm the activity of Wnt5a to activate JNK, we produced recombinant mouse Wnt5a in L cells and obtained Wnt5a in the culture medium. Addition of the Wnt5a-containing medium, but not the control medium, to NIH 3T3 cells resulted in a rapid and significant activation of JNK (Figure 1C). These results indicate that Wnt5a is capable of activating JNK.

Molecular cloning of *Xenopus* JNK and *Xenopus* MKK7

As it is well established that Wnt5a is able to regulate convergent extension movements in *Xenopus* (Moon et al., 1993a; Torres et al., 1996), we then examined whether the MKK7/JNK pathway mediates the action of Wnt5a on these cell movements. To this end, we had to perform molecular cloning of *Xenopus* JNK and *Xenopus* MKK7, which is a direct activator of JNK (Davis, 2000), as these cDNAs were not identified yet. The identified *Xenopus* JNK1 and *Xenopus* MKK7 showed 93 and 81% identity to mouse JNK1 and mouse MKK7, respectively (Figure 2A). Both *Xenopus* JNK1 and *Xenopus* MKK7 were expressed maternally (data not shown). They were strongly expressed in presumptive ectoderm, mesoderm regions, and weakly expressed in endoderm regions during early stages and in head and dorsal regions during neurula and tailbud stages (Figure 2B and C). Because convergent extension during gastrulation occurs at the dorsal side of the embryo, these expression patterns of JNK1 and MKK7 are compatible with their possible role in regulation of convergent extension movements.

The MKK7/JNK pathway mediates the action of Wnt5a

We assessed convergent extension by observing changes in morphology of ectodermal explants stimulated by BVg1. Control explants elongated significantly and underwent typical changes in morphology (Torres et al., 1996; Tada and Smith, 2000) (Figure 3A and B, control). During this process, cells are thought to converge towards the dorsal midline region and intercalate, thereby extending the main body of the explants (Keller et al., 1992; Sokol, 2000). In agreement with the previous results (Moon et al., 1993a; Torres et al., 1996), expression of Wnt5a inhibited both elongation and morphological changes of explants (Figure 3A and B, Wnt5a), confirming that Wnt5a has an activity to regulate convergent extension movements. Remarkably, co-expression of a dominant-negative form of *Xenopus* JNK1, JNK APF, markedly canceled this inhibitory effect of Wnt5a; the explants elongated and underwent typical morphological changes (Figure 3A and B, Wnt5a + JNK APF). Likewise, co-expression of a dominant-negative form of *Xenopus* MKK7, MKK7 3A, canceled the effect of Wnt5a (Figure 3A and B, Wnt5a + MKK7 3A). Expression of JNK APF alone (data not shown) or MKK7 3A alone (see Figure 4D and E, MKK7 3A) in the absence of Wnt5a did not affect significantly

Fig. 1. JNK is activated by Wnt5a. (A) An expression plasmid of HA-tagged MAPK family (HA-JNK2, HA-ERK2, or HA-p38α) was cotransfected with an indicated expression plasmid into NIH 3T3 cells. The activity of HA-MAPKs was measured. The amounts of immunoprecipitated HA-MAPKs were detected by immunoblotting with rabbit anti-HA antibody (αHA). (B) The histogram shows average relative JNK activities from four independent experiments. The activity of the control was set at 1. Error bars represent mean ± SD. In the Student's *t*-test, the activation of JNK by Wnt5a is statistically significant ($P < 0.05$). (C) NIH 3T3 cells were harvested at 15, 30 and 60 min after addition of Wnt5a-containing medium or control medium, and analyzed for JNK activation by the kinase assay. At time point 60 min, the activity of JNK in Wnt5a was ~2-fold higher than that in the control medium ($P < 0.05$, in the Student's *t*-test).

the elongation of the explants. Co-expression of wild-type JNK or wild-type MKK7 with Wnt5a did not affect significantly the Wnt5a-induced inhibition of the convergent extension of explants (Figure 3B). It should be noted that dominant-negative JNK or dominant-negative MKK7 did not affect the expression of mesodermal markers such as α-actin and Xbra (Figure 3C), suggesting that these constructs specifically affect the Wnt5a signaling pathway. In addition, our data indicate that Wnt8, which belongs to another Wnt subfamily, had essentially no effect on convergent extension movements (Figure 3B, Wnt8). This result is also consistent with the previous results (Torres et al., 1996). All these results taken together suggest that the effect of Wnt5a on the convergent extension movements is mediated through the activation of the JNK pathway.

The MKK7/JNK pathway regulates convergent extension movements

We then examined whether activation of the JNK pathway alone is capable of inhibiting convergent extension movements. While expression of wild-type JNK did not inhibit elongation or morphological changes of the explants (Figure 4A and B, JNK

Fig. 2. Molecular cloning of *Xenopus* JNK1 and *Xenopus* MKK7. (A) Alignments of *Xenopus* JNK1 and *Xenopus* MKK7 with their mouse homologs. Inverted amino acids indicate conserved amino acids. (B) Whole-mount *in situ* hybridization analysis of *Xenopus* JNK1 and *Xenopus* MKK7. (C) RT-PCR analysis of expression of *Xenopus* MKK7 and *Xenopus* JNK1. Dorsal mesoderm (DM), ventral mesoderm (VM), ectoderm (ecto) and endoderm (endo) regions were dissected from 10 embryos as shown (left panel, stage 11). Head, dorsal and ventral regions were dissected from five embryos as shown (right panel, stage 24). Each was processed for RT-PCR. The dorsal mesoderm marker Chordin, the endoderm marker Xsox17α, the ventral mesoderm marker Xwnt8, the pan-mesodermal marker Xbra, the forebrain marker Otx2 and the somitic muscle marker muscle actin were also analyzed. *Xenopus* embryonic ornithine decarboxylase (XeODC) is a loading control. The DDBJ/EMBL/GenBank accession No. for *Xenopus* JNK1 is AB073999 and for *Xenopus* MKK7 is AB074001.

WT), co-expression of constitutively active MKK7, MKK7 DED, significantly inhibited the changes (Figure 4A and B, JNK WT + MKK7 DED). Expression of MKK7 DED alone also inhibited the changes (Figure 4D and E, MKK7 DED). Expression of MKK7 3A did not inhibit markedly these morphogenetic movements (Figure 4A and B, JNK WT + MKK7 3A; Figure 4D and E, MKK7 3A). It was confirmed that these treatments did not affect the expression of mesodermal markers (Figure 4C and F). Thus, activation of the JNK pathway is capable of inhibiting specifically convergent extension movements. We then tested the effect of activation of the JNK pathway in whole embryos. Expression of constitutively active MKK7 plus wild-type JNK, but not that of dominant-negative MKK7 plus wild-type JNK, into dorsal marginal zones in 4-cell embryos resulted in incomplete gastrulation, and consequently the embryos showed a shortened anterior-posterior axis (Figure 4G and H). Injection of these mRNAs into ventral marginal zones did not cause any defect (data not shown). To examine whether JNK is necessary for convergent extension in *Xenopus* embryos, we used the morpholino oligo (MO) against JNK. Immunoblotting data showed that the JNK

MO, but not the control MO, reduced the protein level of JNK to approximately one-fifth (Figure 4I). Thus, we injected the JNK MO into dorsal marginal zones in 4-cell embryos. The embryos injected with the JNK MO showed incomplete gastrulation and the embryos showed a shortened anterior-posterior axis (Figure 4I, upper), while embryos injected with the control MO were normal (Figure 4I, lower). These defects appear to result from the inhibition of convergent extension during gastrulation. These results taken together suggest that either hyperactivation of JNK or depletion of JNK inhibits the correct convergent extension movements. This is consistent with the previous results in which overexpression of either wild-type Dishevelled or presumable dominant negative forms of Dishevelled, Dsh ΔDEP or Dsh ΔPDZ, inhibits the convergent extension in *Xenopus* (Heisenberg *et al.*, 2000), and either overexpression of wild-type Dishevelled or the loss of function allele of Dishevelled results in defects in the *Drosophila* PCP (Boutros *et al.*, 1998). Thus, it is likely that the appropriate activation of JNK is necessary for both the PCP in *Drosophila* and the convergent extension movements in vertebrates.

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Fig. 3. Effect of Wnt5a on convergent extension movements is mediated through the MKK7/JNK pathway. (A and B) Animal caps expressing indicated constructs with BVg1. Each combination of RNA (Wnt5a, 100 pg; Wnt8, 100 pg; JNK WT, 2 ng; JNK APF, 2 ng; MKK7 WT, 2 ng; MKK7 3A, 2 ng; MKK7 DED, 2 ng) with BVg1 RNA (50 pg) was injected into the animal pole region of two cells of 4-cell stage embryos. Each animal cap was cultured to stage 19. Typical results are shown in (A). The percentages of elongated animal caps are shown in (B). –, no elongation; +/-, partial elongation; +, strong elongation. $n = 34$ –76 for each condition. (C) RT-PCR detecting expression of mesodermal markers in animal caps derived from embryos expressing indicated constructs together with BVg1. Markers were analyzed at stage 19.

DISCUSSION

In this study, we have shown that Wnt5a is able to activate JNK and the activated JNK is able to function downstream of Wnt5a to regulate convergent extension movements in *Xenopus*.

Our results also suggest that the appropriate activation of JNK is necessary for correct convergent extension. It has previously been shown that overexpressed Dishevelled inhibits the convergent extension (Wallingford *et al.*, 2000). In our experiments, the antisense JNK MO canceled significantly the overexpressed Dishevelled-induced inhibition of the convergent extension movements of explants (our unpublished observations), suggesting that Dishevelled lies upstream of JNK. Since recent reports indicated that in zebrafish and *Xenopus* embryos, the activity of Wnt11, a member of the Wnt5a class ligands, is required for cells to undergo correct convergent extension movements (Heisenberg *et al.*, 2000; Tada and Smith, 2000), the JNK pathway *in vivo* may lie downstream of Wnt11 rather than Wnt5a, or both Wnt5a and Wnt11 may function redundantly. In any case, it can be concluded that the non-canonical Wnt/JNK pathway is conserved evolutionarily in both vertebrates and invertebrates, as previous studies demonstrated that a pathway regulating convergent extension in developing vertebrate embryos is equivalent to the PCP pathway (= the non-canonical Wnt pathway) in *Drosophila* (Heisenberg *et al.*, 2000; Sokol, 2000; Tada and Smith, 2000; Wallingford *et al.*, 2000), and that the PCP pathway in *Drosophila* signals via the JNK pathway to control cell polarity (Boutros *et al.*, 1998). The mechanism by which JNK regulates convergent extension movements in vertebrates remains to be established. Our preliminary data suggest that activated JNK affects cell–cell adhesion (our unpublished observations). This is in good agreement with the previous report indicating that Wnt5a is able to decrease the cell–cell adhesion (Torres *et al.*, 1996). Furthermore, a study in *Drosophila* suggested a role for the transcription factor c-Jun, one of major targets of JNK, in the PCP pathway (Weber *et al.*, 2000). It is thus possible that the JNK/c-Jun-mediated gene expression is also important for regulation of the morphogenetic cell movements. Identifying molecular components upstream and downstream of the MKK7/JNK cascade in the non-canonical Wnt pathway in vertebrates is a priority for future research.

METHODS

Plasmid construction. *Xenopus* Wnt5a (Xwnt5a) and *Xenopus* Wnt8 (Xwnt8) cDNAs were isolated by PCR amplification and cloned into a modified pCS2-Myc vector (pCS4-Myc) (Moriguchi *et al.*, 1999). cDNAs for *Xenopus* JNK1 and *Xenopus* MKK7 were isolated from a *Xenopus* oocyte cDNA library by plaque hybridization, and cloned into an sp64T vector. A dominant-negative *Xenopus* JNK1 (JNK APF) was constructed by replacing Thr183 and Tyr185 with alanine and phenylalanine, respectively. A constitutively active *Xenopus* MKK7 (MKK7 DED) was constructed by replacing Ser268, Thr272 and Ser274 with aspartic acid, glutamic acid and aspartic acid, respectively. A dominant-negative *Xenopus* MKK7 (MKK7 3A) was constructed by replacing Ser268, Thr272 and Ser274 with alanines. These mutations were made by site-directed mutagenesis (Quick Change™; Stratagene) and confirmed by DNA sequencing.

Cell culture, transient transfection and Wnt5a-containing medium. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Transfection into cells was done by the LipofectAMINE™ method with the PLUS reagent according to the manufacturer's

instructions (Life Technologies) with the use of 2.0 µg of total DNA/35 mm dish. The detailed procedure for preparing mouse Wnt5a-containing medium will be described elsewhere (R. Takada, H. Kondoh and S. Takada, in preparation). Briefly, a stably transfected L cell clone, which secreted Wnt5a protein into the medium efficiently, was used to prepare Wnt5a-containing medium, while L cells, which were stably transfected with a control vector, were used to prepare control medium in this study.

Kinase assays. Eighteen hours after transfection, NIH 3T3 cells were lysed, and the activity of HA-tagged protein kinases was determined by the immune complex kinase assay as described previously (Moriguchi et al., 1996, 1997). To determine the amounts of HA-tagged proteins, the immunoprecipitates were subjected to immunoblotting with rabbit anti-HA-antibody (Y-11; Santa Cruz Biotechnology). For kinase assays of JNK stimulated by Wnt5a-containing medium, endogenous JNK was immunoprecipitated with goat anti-JNK1 antibody (C-17; Santa Cruz Biotechnology). Then, the kinase assay was performed as described above.

Embryonic manipulations and microinjection. Embryos were *in vitro* fertilized, dejellied and cultured in 0.1 MBS [1.5 mM HEPES pH 7.4, 8.8 mM NaCl, 0.1 mM KCl, 0.24 mM NaHCO₃, 0.082 mM MgSO₄, 0.03 mM Ca (NO₃)₂ and 0.041 mM CaCl₂]. *In vitro* synthesis of capped mRNA was performed using the Ambion mMESSAGE mMACHINE kit. The RNAs were injected into 4-cell stage embryos. Animal caps were excised from stage 9 embryos and cultured in 1× Steinberg solution. Animal caps expressing BVg1 (control explants) elongated significantly and underwent typical morphological changes (see text). Whole-mount *in situ* hybridization was performed as described (Masuyama et al., 1999).

RT-PCR. Total RNA was isolated from 10 animal caps or five whole embryos by using TRIzol (Gibco-BRL) according to the manufacturer's instructions. RT-PCR were performed as described (Masuyama et al., 1999). Primers for Xbra, EF1α, muscle actin, Otx2, Xsox17α, Chordin, Xwnt8 and XeODC have been described elsewhere (Hudson et al., 1997; Shibuya et al., 1998; Masuyama et al., 1999). The sequences of other primer pairs used were follows: XeMKK7 [forward (f), 5'-CGGAGAA-GAATCGAGCTGGAG; reverse (r), 5'-CTTGACCGCAAGTC-CCGCTGC], XeJNK [f, 5'-CCAAGAGAGCTTATCGGGAAC; r, 5'-TCCCAAGATGACTTCTGGAGC].

Morpholino oligos. The antisense oligodeoxynucleotide used was a 25mer morpholino oligo (Gene Tools LLC) with the base composition 5'-TGCTGTACGCTTGCTTCGGCTCAT-3' (JNK MO). We used the inverted antisense as a control (control MO). Oligos were resuspended in sterile, filtered water and injected. In the experiment shown in Figure 4J, animal caps were dissected from stage 8.5 embryos that had been injected with the JNK MO or the control MO at 4-cell stage. The MO had been injected into the animal hemisphere. Each of the five animal caps was cultured for 18 h after dissection, crushed by pipetting in a buffer (100 µl) containing 50 mM Tris-HCl pH 7.5, 25 mM 2-glycerophosphate, 10 mM EGTA, 2 mM MgCl₂, 2 mM dithiothreitol, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride and 0.2% aprotinin, and then centrifuged at 15 000 g for 30 min. The supernatant was used for immunoblotting with rabbit anti-JNK1 antibody (C-17; Santa Cruz Biotechnology) and rabbit anti-*Xenopus* ERK antibody (Adachi et al., 2000).

ACKNOWLEDGEMENTS

We thank Dr Douglas A. Melton for BVg1 and K. Kawachi and other members of our laboratory for technical comments and helpful discussions. This work was supported by grants from the Ministry of Education, Science and Culture of Japan (to E.N.).

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DOI: 10.1093/embo-reports/kvf008