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
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

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
JNK in the non-canonical Wnt pathway

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.

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.
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
Fig. 4. Appropriate activation of JNK is necessary for correct convergent extension movements. (A) Animal caps expressing indicated constructs with BVg1. Each combination of RNA (JNK WT, 500 pg; MKK7 DED, 500 pg; MKK7 3A, 500 pg) 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. (B) Histogram showing the percentages of elongated animal caps. –, no elongation; +/-, partial elongation; +, strong elongation. n = 65–100 in each condition. In the χ^2 test, the difference between ‘control’ and ‘JNK WT plus MKK7 DED’ is statistically significant (P < 0.01). (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. (D) Animal caps expressing indicated constructs with BVg1. Each combination of RNA (MKK7 DED, 500 pg; MKK7 3A, 500 pg) with BVg1 RNA (50 pg) was injected into the animal pole region of four cells of 4-cell stage embryos. Each animal cap was cultured to stage 19. (E) Histogram showing the percentages of elongated animal caps. –, no elongation; +/-, partial elongation; +, strong elongation. n = 46–51 in each condition. (F) 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. (G) Control embryos injected with water (top), embryos injected with JNK WT RNA (500 pg) plus MKK7 DED RNA (500 pg) (middle) and embryos injected with JNK WT RNA (500 pg) plus MKK7 3A RNA (500 pg) (bottom). Each embryo was cultured to stage 14. Eighty-one percent of the embryos injected with JNK WT plus MKK7 DED (n = 59) showed the defect in gastrulation movements, while only 20% of the embryos injected with JNK WT plus MKK7 3A (n = 60) showed the defect. (H) Control embryos injected with water (top), embryos injected with JNK WT RNA (250 pg) plus MKK7 DED RNA (250 pg) (middle) and embryos injected with JNK WT RNA (250 pg) plus MKK7 3A RNA (250 pg) (bottom). Each embryo was cultured to stage 34. Fifty percent of the embryos injected with JNK WT plus MKK7 DED (n = 56) showed a markedly shortened body axis, while only 18% of the embryos injected with JNK WT plus MKK7 3A (n = 51) showed the shortened body axis. (I) The JNK MO (80 ng, upper) or the control MO (80 ng, lower) was injected into the dorsal marginal zone at 4-cell stage embryos. Each embryo was cultured to stage 34. Seventy percent of the embryos injected with the JNK MO (n = 35) showed a markedly shortened body axis, while only 20% of the embryos injected with the control MO (n = 40) showed the shortened body axis. (J) The protein level of JNK is greatly decreased in the JNK MO injected embryos. For details see Methods.
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 Lcels, 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 XedOC have been described elsewhere (Hudson et al., 1997; Shibuya et al., 1997; Masuyama et al., 1999). The sequences of other primer pairs used were as follows: XeMKK7 [forward (f), 5′-CGGAGAAGAATCGAGCTGGAG; reverse (r), 5′-CTTGAGCCCAATGTCGCGTGC], XeJNK1 [f, 5′-CCAAGAGAGCTATCGGAAGAC; r, 5′-TCCAAAGATGATCCTTGGAGC].

Morphinolo oligos. The antisense oligodeoxynucleotide used was a 25mer morphinolo oligo (Gene Tools LLC) with the base composition 5′-TGCTGTACGGCTGCTGTCGGCTAC-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-mercaptoethanol, 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).

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