The Role of Heterodimeric AP-1 Protein Comprised of JunD and c-Fos Proteins in Hematopoiesis*1

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Background: AP-1 (activator protein-1) is a transcription factor comprised of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) family members.

Results: AP-1 comprised of JunD and c-Fos induces hematopoietic gene expression and is regulated by BMP-4.

Conclusion: AP-1JunD/c-Fos has a crucial role in hematopoiesis and is required for BMP-4-induced hematopoiesis.

Significance: This work provides new insights regarding the role of AP-1 in hematopoiesis.

Activator protein-1 (AP-1) regulates a wide range of cellular processes including proliferation, differentiation, and apoptosis. As a transcription factor, AP-1 is commonly found as a heterodimer comprised of c-Jun and c-Fos proteins. However, other heterodimers may also be formed. The function of these dimers, specifically the heterodimeric AP-1 comprised of JunD and c-Fos (AP-1JunD/c-Fos), has not been elucidated. Here, we identified a function of AP-1JunD/c-Fos in Xenopus hematopoiesis. A gain-of-function study performed by overexpressing junD and c-fos and a loss-of-function study using morpholino junD demonstrate a critical role for AP-1JunD/c-Fos in hematopoiesis during Xenopus embryogenesis. Additionally, we confirmed that JunD of AP-1JunD/c-Fos is required for BMP-4-induced hematopoiesis. We also demonstrated that BMP-4 regulated JunD activity at the transcriptional regulation and post-translational modification levels. Collectively, our findings identify AP-1JunD/c-Fos as a novel hematopoietic transcription factor and the requirement of AP-1JunD/c-Fos in BMP-4-induced hematopoiesis during Xenopus hematopoiesis.

Activator protein-1 (AP-1)3 is an evolutionarily conserved bZip family transcription factor composed of Jun family members (e.g. c-Jun, JunB, and JunD) and Fos family members (e.g. c-Fos, FosB, Fra-1, and Fra-2). Whereas the Fos proteins can only heterodimerize with members of the Jun family, the Jun proteins can both homodimerize and heterodimerize with Fos members to form transcriptionally active complexes (1). Each of the AP-1 components is differentially expressed and regulated to perform subtly different functions (2). Moreover, different combinations of AP-1 components have been implicated in a large variety of biological processes, including proliferation, differentiation, apoptosis, and development (2–5). However, despite increasing knowledge regarding the physiological functions of AP-1, a specific role for distinct AP-1 components in early embryogenesis has only been partially elucidated (5–8).

In the Xenopus embryo, two sites of hematopoiesis are present and include the ventral blood island (VBI), which is analogous to the yolk sac blood islands of higher vertebrates, and the dorsal lateral plate region that is analogous to the aorta, gonads and mesonephros region (AGM) of vertebrates (9). Specifically, the VBI is located on the ventral side of the embryo, and the hematopoietic stem cells of the VBI differentiate primarily into embryonic erythroid cells (primitive hematopoiesis) (9, 10).

BMP-4 (bone morphogenetic protein-4) and transforming growth factor-β (TGF-β) are known to be required during gastrulation for specifying the ventral character of the embryonic mesoderm and are therefore candidates for controlling the development of VBI-derived blood progenitors (11, 12). Ectopic expression of BMP-4 in animal cap explants induces expression of the hematopoietic-specific transcription factor, SCL (stem cell leukemia), GATA-1, GATA-2, and GATA-3, LMO2, Neptune, and globin blood marker (11, 13, 14).

Here, we characterize the novel function of heterodimeric AP-1 comprised of junD and c-fos (AP-1JunD/c-Fos) in hematopoiesis during Xenopus development. Furthermore, we provide evidence showing that AP-1JunD/c-Fos function is required for BMP-4-induced hematopoiesis. In addition, we demonstrate that BMP-4 controls AP-1JunD/c-Fos activity through transcriptional and post-translational regulation of junD during hematopoiesis.
EXPERIMENTAL PROCEDURES

Xenopus Embryo Manipulations and Animal Cap Assay—All procedures were followed as described previously (15).

Plasmid Constructs and In Vitro Transcription—XjunD (Genbank™ accession no. BC079782) and X-cfos (Genbank™ accession no. BC079689) cDNAs were isolated from Xenopus and were inserted into the EcoRI/XbaI site of the pCS2 (+) vector and the FLAG- or HA-tagged pCS2 (+) vector by PCR. For examining XjunD-MO specificity, the XjunD cDNA was cloned into the pCS2-HA vector at the BamHI/EcoRV site. For rescue experiments, mouse junD cDNA was cloned into the pcDNA3.1 (+) vector. Capped mRNAs were synthesized from linearized vectors using with Ambion mRNA Machine kit. MOs was used and control MO (5'-H11032), provided by GeneTools was used as a control.

Additionally, we examined whether AP-1JunD/c-Fos could regulate the transcription of SCL using an SCL promoter-luciferase reporter gene. AP-1JunD/c-Fos enhanced the promoter activity of this gene (Fig. 1C) but not the activity of SCL-mAP-1-luciferase, which is mutated at the consensus AP-1 binding site, suggesting the involvement of AP-1 binding site, suggesting the involvement of AP-1JunD/c-Fos in the transcriptional regulation of the SCL gene.

To further determine whether AP-1JunD/c-Fos is indeed necessary for hematopoiesis in Xenopus laevis development, we performed a loss-of-function study using morpholino antisense directed against junD (MO-junD). We generated two types of antisense morpholino oligonucleotides (MO1 and MO2) capable of depleting the XjunD protein. The MO-junDs were effective in specifically reducing the level of the 3'-untranslated region of junD mRNA (supplemental Fig. 2A). qRT-PCR analysis showed that the junD-depleted embryos repressed the expression of SCL and globin compared with control embryos without affecting the dorsal mesoderm marker, actin. Additionally, co-injection of mRNA of mouse junD (mjJunD) rescued these markers that had been repressed by MO-junDs (Fig. 1E).

RESULTS

AP-1JunD/c-Fos Has a Role in Hematopoiesis—To define the function of AP-1JunD/c-Fos in hematopoiesis, we analyzed the gene expression profile induced by AP-1JunD/c-Fos in embryonic stem cells of Xenopus animal caps explants. Co-expression of junD and c-fos induced hematopoietic transcription factors, GATA-1, SCL, LMO-2, and Neptune, which are involved in the initial blood program (Fig. 1A), and α-globin, the erythrocyte specific marker, in a dose-dependent manner (Fig. 1B). Otherwise, junD or c-fos mRNA alone could not induce globin (supplemental Fig. 1). Hematopoietic transcription factors such as GATA-1, SCL, LMO-2, and Neptune alone do not induce hematopoietic cells from the embryonic ectoderm. Interestingly, AP-1JunD/c-Fos alone is sufficient to induce blood cell formation from the ectoderm similar to BMP-4 signaling.

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Whole Mount in Situ Hybridization—Embryos were injected with the indicated mRNAs and then processed for whole-mount in situ hybridization by using standard methods (16) with α-globin probes.

Western Blot Analysis—All procedures were followed as described previously (15). For Western blots, anti-HA-peroxidase conjugate (Roche Applied Science), anti-phospho-Jun (α-73) (Cell Signaling, Danvers, MA), and monoclonal anti-actin (Sigma) were used. Proteins were visualized using ECL Western blotting detection reagents (Amersham Biosciences).

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The control MO-injected embryos had no effect on the expression of these markers (supplemental Fig. 2B). Furthermore, XjunD- or control MO were injected into one blastomere of two-cell embryos, together with the lineage tracer β-galactosidase (200 pg; Fig. 1F). Embryos were cultured until stage 32 and stained for β-galactosidase activity to confirm the accuracy of injections (blue staining, Fig. 1, G and H), followed by in situ hybridization to detect expression of globin, a marker of differentiated red blood cells (RBCs) (purple staining, Fig. 1, G and H). Embryos injected with MO-junD appeared normal but showed a severe repres-
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FIGURE 1. AP-1JunD/c-Fos Is Required for the BMP-4-induced Hematopoiesis—BMP-4 is a TGF-β family member that has been shown to be an essential factor in the differentiation of primitive blood cells in the X. laevis embryo. To further verify the role of AP-1JunD/c-Fos in hematopoiesis, we investigated whether the role of AP-1JunD/c-Fos in blood formation is associated with BMP-4. First, we determined whether AP-1JunD/c-Fos and BMP-4 have synergistic effects in hematopoiesis. For analyzing the synergistic effect between AP-1 and BMP-4, we used a low dose of mRNA. A low dose of AP-1JunD/c-Fos or BMP-4 alone had no apparent effect on the expression of hematopoietic markers (Fig. 3A). As indicated earlier, for hematopoietic markers and globin expression, a high dose of BMP-4 mRNA is required in animal cap explants. A combination of AP-1JunD/c-Fos and BMP-4 resulted in a marked increase in the level of hematopoietic markers, including globin (Fig. 3A). The expression of hematopoietic markers and globin induced by the combination of AP-1JunD/c-Fos and BMP-4 is higher than the additive value of each alone. To further verify the role of AP-1JunD/c-Fos in hematopoiesis, we performed a cell lineage tracing experiment. Embryos at the eight-cell stage were injected with GFP mRNA alone or together with AP-1 mRNA (junD and c-fos) into animal blastomeres at the dorsal portion of the embryos (D1) (Fig. 2A). Two of the dorsal animal blastomeres at the eight-cell stage embryo (D1) differentiated into dorsal and anterior neural tissues. Thus, when GFP mRNA alone was injected into the D1 region, fluorescent cells were detected in dorsal and anterior neural tissues. However, when GFP and a low dose of AP-1JunD/c-Fos mRNAs (500 pg) were co-injected into the D1 regions, fluorescent cells were detected in the VBI region but also remained in the dorsal region (Fig. 2C). The VBI is indicated by in situ hybridization of globin (Fig. 2, B and C, lower panels). When AP-1JunD/c-Fos mRNAs were injected into animal blastomeres of the ventral part of the embryos (V1) at the eight-cell stage as a control experiment (Fig. 2D), GFP was expressed at the ventroposterior epidermis (Fig. 2E, 17, 18). A similar pattern was seen with control embryos injected with GFP mRNA into the ventral part of animal blastomeres (Fig. 2F). These results indicate that junD and c-fos not only induce hematopoiesis but also have the ability to change a part of dorsal fated tissue into VBI tissue in whole embryos.

**AP-1JunD/c-Fos Converts Dorsal-fated Tissue into Ventral-fated tissue, Specifically VBI—**To elucidate the in vivo function of AP-1JunD/c-Fos during Xenopus embryogenesis, we performed a cell lineage tracing experiment. Embryos at the eight-cell stage were injected with GFP mRNA alone or together with AP-1 mRNA (junD and c-fos) into animal blastomeres at the dorsal portion of the embryos (D1) (Fig. 2A). Two of the dorsal animal blastomeres at the eight-cell stage embryo (D1) differentiated into dorsal and anterior neural tissues. Thus, when GFP mRNA alone was injected into the D1 region, fluorescent cells were detected in dorsal and anterior neural tissues. However, when GFP and a low dose of AP-1JunD/c-Fos mRNAs (500 pg) were co-injected into the D1 regions, fluorescent cells were detected in the VBI region but also remained in the dorsal region (Fig. 2C). The VBI is indicated by in situ hybridization of globin (Fig. 2, B and C, lower panels). When AP-1JunD/c-Fos mRNAs were injected into animal blastomeres of the ventral part of the embryos (V1) at the eight-cell stage as a control experiment (Fig. 2D), GFP was expressed at the ventroposterior epidermis (Fig. 2E, 17, 18). A similar pattern was seen with control embryos injected with GFP mRNA into the ventral part of animal blastomeres (Fig. 2F). These results indicate that junD and c-fos not only induce hematopoiesis but also have the ability to change a part of dorsal fated tissue into VBI tissue in whole embryos.

**AP-1JunD/c-Fos Is Required for the BMP-4-induced Hematopoiesis**—BMP-4 is a TGF-β family member that has been shown to be an essential factor in the differentiation of primitive blood cells in the X. laevis embryo.

Together with MO junD (20 ng) or mouse junD (1 ng) (mjunD) as illustrated. G and H, embryos were stained for β-galactosidase (β-gal) activity at stage 30 (blue stain) followed by in situ hybridization of globin expression (purple stain). The expression of globin is repressed in the MO junD-injected side. H, mouse junD mRNA (mjunD) rescues globin expression that is repressed by expression of MO junD, EF1α, loading control; w.e., whole embryo was used as a positive control for PCR. —RT, control reaction without reverse transcriptase; con, animal cap samples obtained from non-injected embryos. ***, p value < 0.001; **, p value < 0.001. IB, immunoblot.
AP-1 confirms this finding, we performed benzidine staining, a specific histochemical stain for differentiated red blood cells. Animal caps derived from embryos injected with \texttt{AP-1}JunD/c-Fos mRNA (500 pg) or \texttt{AP-1} (junD and c-fos, 500 pg) mRNA (C and E) were injected into dorsal animal blastomeres (D1) or ventral animal blastomeres (V1) of eight-cell stage embryos and then cultured until stages 28–30. Embryos were fixed, and GFP expression was observed by green fluorescent microscopy. B and C, dorsally expressed GFP (B, upper panel) is partially transferred into the ventral blood island region (C, three arrows in upper panel). E and F, embryos injected with either \texttt{AP-1} alone (F, upper panel) or together with \texttt{AP-1}JunD/c-Fos (E, upper panel) show a similar expression pattern of GFP, which is expressed at the ventroposterior epidermis. The ventral blood island is indicated by \textit{in situ} hybridization of globin (B–F, lower panel). The number (n) of phenotypes for each group is presented.

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\caption{\textbf{AP-1JunD/c-Fos converts dorsal-fated tissue into ventral-fated tissue (ventral blood island).} A and D, illustration of the scheme of the experiment. GFP mRNA (200 pg, B) or \texttt{AP-1}JunD/c-Fos mRNA (500 pg) were injected into dorsal animal blastomeres (D1) or ventral animal blastomeres (V1) of eight-cell stage embryos and then cultured until stages 28–30. Embryos were fixed, and GFP expression was observed by green fluorescent microscopy. B and C, dorsally expressed GFP (B, upper panel) is partially transferred into the ventral blood island region (C, three arrows in upper panel). E and F, embryos injected with either \texttt{AP-1}alone (F, upper panel) or together with \texttt{AP-1}JunD/c-Fos (E, upper panel) show a similar expression pattern of GFP, which is expressed at the ventroposterior epidermis. The ventral blood island is indicated by \textit{in situ} hybridization of globin (B–F, lower panel). The number (n) of phenotypes for each group is presented.}
\end{figure}

\begin{figure}[h]
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\caption{\textbf{AP-1JunD/c-Fos is required for hematopoiesis induced by BMP-4.} A and B, animal caps, explanted from embryos injected with the indicated mRNAs were incubated until stage 20–24 and used for qRT-PCR analysis (A) or benzidine staining (B). A, \texttt{AP-1}JunD/c-Fos and BMP-4 synergistically induce hematopoietic markers and globin. B, blood formation stained by benzidine is synergistically enhanced by \texttt{AP-1}JunD/c-Fos and BMP-4. C, the activity of the (AP-1)-luciferase reporter gene is synergistically activated by co-injection of \texttt{AP-1}JunD/c-Fos and BMP-4. An (AP-1)-luciferase assay using animal cap explants derived from embryos injected with the (AP-1)-luciferase reporter gene alone or in combination with the indicated mRNA was performed. Luciferase activity was measured at stage 18. Values are averages from at least three independent experiments. RLU, relative luciferase activity. D, animal caps, explanted from embryos injected with the indicated mRNAs or MO \texttt{JunD} (20 ng), were incubated until stage 20–24 and used for qRT-PCR analysis. MO \texttt{JunD} selectively blocks BMP-4-induced expression of globin, LMO2, and SCL. Injection of mouse \texttt{junD} mRNA rescues the BMP-4 induction of globin, LMO2, and SCL as well as the activity of the (AP-1)-luciferase reporter gene in MO \texttt{junD}-injected animal caps (E). EF1\textalpha, loading control; w.e., whole embryo was used as a positive control for PCR; rt, control reaction without reverse transcriptase. **, \textit{p} value < 0.01; ***, \textit{p} value < 0.001.}
\end{figure}

ther confirm this finding, we performed benzidine staining, a specific histochemical stain for differentiated red blood cells. Animal caps derived from embryos injected with \texttt{AP-1}JunD/c-Fos as a control (Fig. 3B, fourth panel) were not stained by benzidine. The low dose of BMP-4-injected animal caps also did not exhibit detectable benzidine staining (Fig. 3B, third panel). However, consistent with the qRT-PCR data, a combination of \texttt{AP-1}JunD/c-Fos and BMP-4 enhanced benzidine staining (Fig. 3B, second panel) compared with each alone (Fig. 3B, first panel). These results indicate a synergistic effect of \texttt{AP-1}JunD/c-Fos and BMP-4 on blood formation.

On the basis of these results, we examined whether BMP signaling acts upstream of \texttt{AP-1}JunD/c-Fos during blood formation. We first determined whether BMP-4 could regulate \texttt{AP-1} activity in an animal cap assay. The activity of the (AP-1)-luciferase reporter gene, which contains four AP-1 binding
**AP-1 Functions in BMP-4-induced Hematopoiesis**

**FIGURE 4. Transcriptional and post-translational modification of AP-1JunD/c-Fos by BMP-4 is required for hematopoiesis.** A, BMP-4 activates the transcription of junD, but not c-fos. ventral marker) was used as a positive control for BMP-4. Animal caps derived from embryos injected with the indicated mRNA were excised and cultured until stage 13 and used for qRT-PCR analysis. con, animal cap samples obtained from non-injected embryos. B, BMP-4 enhances phosphorylation of JunD at serine 67; in contrast, dominant-negative BMP receptor inhibits the phosphorylation of JunD. Embryos injected with wild-type HA-junD (W) or mutant HA-junD (M2) alone or in combination with 1 ng of BMP-4 or dominant-negative BMP receptor mRNA were used for Western blotting. Phosphorylation of JunD was analyzed by Western blot using anti-phospho-Jun (α-73). Western blotting with anti-HA shows that equal amounts of expressed JunD were loaded. C, band density was measured using the NIH ImageJ program. D-F, animal caps, explanted from embryos injected with the indicated mRNAs or in combination with the (AP-1)4-luciferase reporter gene or SCL-luciferase reporter gene, were used for qRT-PCR analysis (D) and the luciferase assay (E and F). The concentration of each mRNA injected into embryos was 1 ng. D, AP-1M2JunD/c-Fos shows a lower induction of hematopoietic markers and globin, compared with AP-1JunD/c-Fos. E and F, the AP-1- and SCL-luciferase activities are consistent with D. Data are shown as means ± S.D. of values from at least three independent experiments. RLU, relative luciferase activity. **, p value < 0.01; ***, p value < 0.001.

sequences (TGAC/GTCA), was enhanced by AP-1 (junD and c-fos) or BMP-4 alone. These results suggest that BMP-4 regulates AP-1JunD/c-Fos activity.

Additionally, co-injection of AP-1 and BMP-4 caused a synergistic effect on the activation of AP-1 (Fig. 3C). This result is consistent with the synergistic effect of AP-1JunD/c-Fos and BMP-4 on blood formation of Xenopus embryos. To confirm whether AP-1JunD/c-Fos is indeed necessary for BMP-4-induced blood formation, we investigated whether depletion of junD could inhibit the expression of BMP-4-induced hematopoietic markers. MO-junD (Fig. 3D) but not control MO (supplemental Fig. 3) effectively inhibited the BMP-4-induced expression of hematopoietic transcription markers (SCL and LMO2), including globin. Consistent with this result, the activity of the AP-1 reporter gene that was enhanced by BMP-4 was suppressed by depletion of junD (Fig. 3E, lanes 1–3). Additionally, the expression of hematopoietic markers and the activity of the AP-1 reporter gene, which were suppressed by MO-junD in the presence of BMP-4, were effectively rescued by co-overexpression of wild-type junD mRNA (Fig. 4, D and E). Taken together, these data support the idea that AP-1JunD/c-Fos is required for BMP-4-mediated hematopoiesis during X. laevis development.

**Phosphorylation and Transcriptional Regulation of JunD by BMP-4 Is Important for the Function of AP-1JunD/c-Fos in Hematopoiesis—**As demonstrated above, the biological ability of AP-1JunD/c-Fos is required for BMP-4-induced hematopoiesis. To study whether BMP-4 regulates AP-1JunD/c-Fos in the embryo, we investigated transcriptional regulation of AP-1JunD/c-Fos by BMP-4. Interestingly, BMP-4 increased the transcription of junD, but not c-fos (Fig. 4A). Xvent1 was used for positive control of BMP-4 (19, 20). Additionally, we investigated whether BMP-4 could phosphorylate JunD in embryos. Serine phosphorylation is a mechanism for regulating AP-1-dependent gene transcription (21, 22). A phospho-Jun antibody (α-73) was used for recognizing junD only if it is phosphorylated at serine 100 (23, 24). To examine phosphorylation of junD by BMP-4, we generated HA-tagged wild-type junD and mutant (M2) junD in which Ser-66, a sequence conserved with mouse junD, was replaced with alanine. Phosphorylation of Ser-66 of JunD was detected using a phospho-Jun antibody (α-73). BMP-4 stimulation enhanced JunD phosphorylation at Ser-66, whereas dominant-negative BMP receptor efficiently inhibited phosphorylation of JunD (Ser-66) (Fig. 4B). The M2 mutant was not affected by BMP-4 or dominant-negative BMP receptor (Fig. 4B). The band density was measured using the NIH ImageJ program (Fig. 4C). This result indicates that BMP-4 is sufficient to regulate Ser-66 phosphorylation of JunD.

To study the biological role of Ser-66 phosphorylation in hematopoiesis of the X. laevis embryo, we compared the activity of mutant M2 junD and wild-type junD in hematopoiesis of Xenopus embryos. Compared with wild-type junD and c-fos (AP-1JunD/c-Fos), the mutant junD (M2) and c-fos
The transcription factor AP-1 is composed of different possible dimer combinations formed between the Jun and Fos family of “bZip” transcription factors. These different AP-1 dimers exhibit similar DNA binding specificities (TGAC/GTCA) but have differences in their transactivation efficiencies (5–8), indicating that distinct AP-1 dimers differentially regulate AP-1 target genes. Thus, we hypothesized that the composition of AP-1 would determine the expression of specific target genes and allow prediction of the nature of the dimeric combinations, which are physiologically relevant under various conditions.

Previously, we have shown that AP-1 is involved in mesoderm induction as a major downstream mediator of FGF signaling (25, 26). Moreover, we demonstrated that heterodimeric AP-1, comprised of c-Jun and c-Fos, mediated activin-induced Spemann organizer gene expression (15). We also reported the involvement of AP-1 in BMP-4 signaling and in BMP-4 expression (26, 27). Although the role of other AP-1 components has not yet been elucidated in X. laevis development, the physiological function of heterodimeric AP-1 comprised of JunD and c-Fos has not yet been elucidated in X. laevis development. In the present study, we elucidated the function of heterodimeric AP-1 comprised of JunD and c-Fos in hematopoiesis of X. laevis development.

To determine the specificity of junD in BMP-4-induced hematopoiesis, we examined whether c-jun had an effect in BMP-4-induced hematopoiesis. Depletion of c-jun using MO c-jun had no effect on BMP-4-induced hematopoiesis; in contrast, MO c-jun generally inhibited activin-induced gene expression (15), suggesting a specific biological function of AP-1junD/c-Fos downstream of BMP-4 (supplemental Fig. 5). Additionally, depletion of junD using MO junD had no effect on activin-induced dorsal mesoderm and endoderm formation (supplemental Fig. 6). Taken together, these results indicated that c-Jun and JunD have distinct roles downstream of activin and BMP-4, suggesting that specific AP-1 composition regulated by diverse signaling determines the expression of specific target genes.

Transcription factors are tightly regulated at the transcriptional, post-transcriptional, and post-translational levels. In the current studies, we discovered that AP-1junD/c-Fos is regulated downstream of BMP-4 signaling at the transcriptional and post-translational levels, and the regulation is important for AP-1junD/c-Fos-induced hematopoiesis. Serine phosphorylation is a representative mechanism for regulating AP-1-dependent gene transcription (21–22). Although numerous studies on the phosphorylation of c-Jun have been reported, JunD phosphorylation and its possible regulatory function have not been well characterized. Previously, JunD has been shown to be phosphorylated in vivo and in vitro (23–24, 28–31). The N terminus of junD contains three sites for MAP kinase phosphorylation (serines 90 and 100 and threonine 117), which are essentially identical to the well characterized regulatory phosphorylation sites of c-Jun (serines 63 and 73 and threonine 91). These sites share a conserved sequence with serines 56 and 66 and threonine 83 of Xenopus JunD. Generally, phosphorylation of serine 100 of JunD was studied to examine JunD activity because the phospho-Jun antibody (α-73) recognizes phosphorylated serine 100 of JunD. Thus, in the present study, we confirmed that phosphorylation of serine 66 of Xenopus JunD, which is identical to serine 100 of mouse JunD, was also detected with the phospho-Jun antibody (α-73). Results indicated that BMP-4 signaling could regulate phosphorylation of serine 66 of Xenopus JunD. However, the kinase activated by BMP-4 that is responsible for phosphorylating JunD at serine 66 remains to be identified in future studies (supplemental Fig. 4).

Additionally, we characterized the biological role of serine 66 of XJunD in hematopoiesis. Overexpression of the phosphorylation mutant form of AP-1junD/c-Fos partially retained activity for its biological role in hematopoiesis, suggesting that other phosphorylation sites might also contribute to the complete biological function of AP-1junD/c-Fos in hematopoiesis.

In the present study, we suggested a new function for AP-1 in hematopoiesis during Xenopus development providing additional evidence of a specific role for individual AP-1 members in distinct cellular process. Furthermore, we suggest that AP-1junD/c-Fos functions downstream of BMP-4 signaling and BMP-4 alters AP-1junD/c-Fos function at the post-translational level by phosphorylation of JunD at serine 66.

Acknowledgment—We thank Ms. Tonya Poorman for help submitting our manuscript.

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