The Extracellular Adenosine Deaminase Growth Factor, ADGF/CECR1, Plays a Role in Xenopus Embryogenesis via the Adenosine/P1 Receptor*

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Adenosine deaminase-related growth factors (ADGF), also known as CECR1 in vertebrates, are a novel family of growth factors with sequence similarity to classical cellular adenosine deaminase. Although genes for ADGF/CECR1 have been identified in both invertebrates as well as vertebrates, their in vivo functions in vertebrates remain unknown. We isolated cDNA clones for two cerc1s from Xenopus laevis. Both recombinant Xenopus CECR1s exhibited adenosine deaminase activity and growth factor activity, and the adenosine deaminase activity was found to be indispensable for growth factor activity. The Xenopus cerc1s are expressed in the somites, pronephros, eyes, cement gland, neural tube, and neural floor plate of the embryos. Knock-down of these two genes using morpholino oligonucleotides caused a reduction in the body size of the embryos, accompanied by selective changes in the expression of developmental marker genes. Injection of adenosine, agonists for adenosine/P1 receptors, or adenosine deaminase inhibitor into late gastrula archenteron embryos resulted in developmental defects similar to those caused by morpholino oligonucleotide injection. These results show, for the first time, the involvement of CECR1s via the adenosine/P1 receptors in vertebrate embryogenesis via regulation of extracellular adenosine concentrations.

Growth factors play important roles in morphogenesis and organogenesis in various animal species. Of these, the adenosine deaminase-related growth factor (ADGF) family is a novel group of growth factors with sequence similarity to classical adenosine deaminase (ADA) in their carboxyl-terminal regions (1–3). Classical ADA is a cytoplasmic enzyme that catalyzes the deamination of adenosine and 2’-deoxyadenosine to inosine and 2’-deoxyxynosine, respectively (4), and has a wide phylogenetic distribution (5). ADA is involved in purine metabolism and plays an indispensable role in the upkeep of a competent immune system in mammals (6). In contrast, ADGFs are comprised of secretory or transmembrane-type ADGFs (1, 2, 7, 8), with some having growth factor-like activities (3, 9–14).

Insect-derived growth factor (IDGF), the first ADGF to be cloned, is a secretory ADGF purified from the conditioned medium of NIH-Sape-4, an embryonic cell line of Sarcophaga peregrina (flesh fly) (1). IDGF stimulates the growth of NIH-Sape-4 cells with a specific activity comparable with mammalian growth factors. Site-directed mutagenesis has demonstrated that the ADA activity of IDGF is essential for its growth factor activity (3, 15). So far, ADGF families have been reported in various invertebrates. In Drosophila melanogaster (fruit fly), Male-specific IDGF was identified as a transmembrane-type ADGF and is suggested to participate in spermatogenesis (7). Recently, six ADGFs have been identified in Drosophila, one of which (Adgf-A2) encodes Male-specific IDGF (8, 16). In addition, ADGF families have been identified in other fruit flies, sand flies, sea slugs, tsetse flies, mosquitoes, fungi, and slime mold (2).

ADGF families have also been identified in vertebrates, with human CECR1 first identified as a member of the ADGF family (17). The human CECR1 is a candidate gene responsible for “cat eye syndrome” (17). Cat eye syndrome is a human developmental disorder characterized by a variety of congenital defects, including ocular coloboma (hence the name “cat eye”), heart and kidney defects, and mental retardation (18, 19). Cat eye syndrome is associated with duplication of the proximal long arm of human chromosome 22, which contains CECR1, suggesting that the syndrome might be due to the overexpression of CECR1 (20). CECR1 homologues have also been identified in humans, cats, dogs, and mice (21, 22).

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The Role of Xenopus CECR1 in Embryogenesis

the genomes of other vertebrate species, such as chimp, baboon, pig, chicken, frog, zebrafish, and pufferfish (2), indicating that the ADGF/CECR1 genes represent a family of evolutionarily conserved genes. Interestingly, no obvious CECR1 homologue has yet been identified in rodents (20).

ADA activity has been reported for some ADGF/CECRs (9–14), including IDGF (3), where the $K_{\text{cat}}$, $V_{\text{max}}$, and $K_{\text{cat}}$ values are comparable with those of mammalian cytoplasmic ADAs (21). As IDGF was originally identified as a growth factor in an embryonic cell line (1) and ADA activity was shown to be indispensable for growth factor activity (3), it is plausible that the ADGF/CECR1 family plays a role in cell proliferation during morphogenesis and organogenesis via its ADA activity. In Drosofila, the lack of Adg-a leads to precocious metamorphic changes during metamorphosis, including the differentiation of macrophage-like cells, fat body disintegration and developmental delay (16, 22). Transgenic expression of human CECR1 in mice results in abnormal heart and kidney development, suggesting a possible role in mammalian development (23). The in vivo function of the CECR1 family in vertebrates, however, remains unclear, as there is no known CECR1 homologue in mice.

In the present study, we analyzed the molecular properties and in vivo functions of Xenopus CECR1s to increase our understanding of the roles of the ADGF/CECR1 family in vertebrates. The results demonstrate a key role of Xenopus CECR1s in embryonic development via their ADA activity.

EXPERIMENTAL PROCEDURES

Embryos and Their Culture—Xenopus laevis embryos were collected and cultured as described previously (24). The embryos were staged according to the method described by Nieuwkoop and Faber (25).

cDNA Cloning for Xenopus cecr1s—To isolate Xenopus cecr1 cDNA, PCR was performed using Xenopus cDNA library (embryo, stage 30, Stratagene). The nucleotide sequences of the degenerated primers used for the PCR were 5’-GT(G/T)GGI(C/A)(G/A)IGA(A/G)GA(C/T)ACIGG-3’ and 5’-CCA(A/G)TCNAT(T/C)TCICCIGC (G/A)TG(G/A)AA-3’, which correspond to $32^5$VGHEDTG$^{351}$ and $32^5$FHAGETDW$^{362}$, respectively. A 114-bp cDNA fragment was amplified and used as a probe for subsequent cDNA cloning. Two hybridization-positive clones were isolated from 1.3 $\times$ 10^5 clones of a Xenopus embryo (stage 30) cDNA library (Stratagene) by plaque hybridization, and their sequences determined.

Phylogenetic Analysis—Deduced amino acid sequences encoded by the vertebrate CECR1-like genes were aligned using CLUSTAL W (26). The molecular tree was constructed using the neighbor-joining method (27) and displayed using TREEVIEWPPC (28). Accession numbers for each gene are: Hs_CECR1 (AF190746), Ss_CECR1 (AF384216), Gg_CECR1 (AY900277), Dr_CECR1-1 (AF384217), Dr_CECR1-2 (Genomic BX323558), Tn_CECR1-1 (Genomic CAAE01014566), and Tn_CECR1-2 (Genomic CAAE01014691).

Preparation of Recombinant Xl_CECR1s—The recombinant Xl_CECR1-1 and Xl_CECR1–2 proteins were produced in Escherichia coli BL21 star (DE3) using a pET100 directional TOPO expression kit (Invitrogen) as well as in Xenopus A6 cells (American Type Culture Collection). In the first method, the coding regions of Xl_cecr1-1 and -2 were amplified by PCR and subcloned into a pET100/D-TOPO vector. His-tagged fusion proteins were then produced in E. coli. Purification was performed using a HiTrap Chelating HP column (GE Healthcare BioSciences). In the second method, the amplified coding regions of Xl_cecr1-1 and -2 were subcloned and transfected to Xenopus A6 cells with FuGENE 6 transfection reagent (Roche Diagnostics). The recombinant proteins were used for assaying ADA and growth factor activities.

Preparation of Antibodies against Xl_CECR1s—To prepare antibodies against Xl_CECR1-1 and Xl_CECR1–2, the synthetic peptides CEDLAMPEASEREL and CKFIKDLAMN-WKKE, which are specific to Xl_CECR1-1 and Xl_CECR1–2, respectively, were conjugated to keyhole limpet hemocyanin and injected separately into an albino rabbit with Complete Freund adjuvant. After three booster injections with Incomplete Freund adjuvant, each antiserum was affinity-purified using the recombinant Xl_CECR1-1 or -2, produced in E. coli.

Assay of the ADA Activity of Xl_CECR1s—The ADA activity of Xl_CECR1s was assayed as described by Iwaki-Egawa et al. (29). Enzymatic activity was determined by quantifying inosine liberated from adenosine. Inosine is converted by purine nucleoside phosphorylase into hypoxanthine; the latter is then converted into uric acid and diformazan by xanthine oxidase and nitro blue tetrazolium. Thus, the optical density at 540 nm of diformazan was measured. In brief, the recombinant Xl_CECR1s were preincubated for 5 min at 37 °C in a reaction mixture containing 20 mM sodium phosphate buffer (pH 6.8), 1 unit/ml xanthine oxidase, 0.24 unit/ml purine nucleoside phosphorylase, and 1 mM nitro blue tetrazolium. Following incubation, the ADA assay was initiated by adding adenosine, and the mixture was further incubated at 37 °C for 15 min. The reaction was stopped by adding 1/4 volume of 50% acetic acid. The absorption at 540 nm of diformazan was then measured. One unit of ADA activity was defined as the amount of enzyme that produced 1 µmol of inosine/min.

Assay of Growth Factor Activity—The growth factor activity assay was performed as described previously by assessing cell proliferation (7). NIH-Sape-4 cells were cultured in M-M medium (30) at 25 °C, as described previously (31). Briefly, NIH-Sape-4 cells were suspended at a density of 2.5 $\times$ 10^5 cells/ml in M-M medium, and then 100 µl of the cell suspension was poured into each well of a 96-well microtiter plate. Cell proliferation was assessed by measuring the enhancement of cellular metabolism using the Alamar Blue assay, after the addition of 1 ng of recombinant Xl_CECR1-1 and 10 ng of Xl_CECR1–2 (32, 33). The cells were then incubated for 2 days at 25 °C in the presence or absence of the test sample. Following this, 15 µl of Alamar Blue (Alamar Biosciences, Inc.) was added, and changes in the color of the culture medium were determined by measuring $A_{570}$ and $A_{510}$. One unit of growth factor activity was defined as the amount giving half-maximal stimulation of cell proliferation.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted from Xenopus embryos using TRIzol reagent (Invitrogen). RT-PCR was performed using the ProStar
First Strand RT-PCR kit (Stratagene). The oligonucleotide primers used to detect XL_cecr1s mRNA were 5'-CATACT-TCTTCCATCGAGAG-3' (sense) and 5'-ACCAAGAGTAGT-GATGGGCATCT-3' (antisense). These primers are designed to detect both XL_cecr1-1 and XL_cecr1-2. As an internal control, ornithine decarboxylase mRNA was detected using the oligonucleotide primers (5'-GTCAATGAGAGGTGTAGTAGTATC-3' (sense) and 5'-TCTATTCCGTCTCCTGAGCAC-3' (antisense)).

Whole Mount In Situ Hybridization—Whole mount in situ hybridization of *Xenopus* embryos was performed as described previously (34). The XL_cecr1 probe consisted of digoxigenin-labeled RNA corresponding to a 605-bp fragment (+904 to +1508) of XL_cecr1–2 cDNA. The Xmyod probe consisted of digoxigenin-labeled RNA corresponding to a 350-bp fragment (+550 to +605) of Xmyo5. The Xa2a probe consisted of digoxigenin-labeled RNA corresponding to a 457-bp fragment (36). The Xmyf-5 probe consisted of digoxigenin-labeled RNA corresponding to a 405-bp fragment (35). The Xmyf-5 probe consisted of digoxigenin-labeled RNA corresponding to a 350-bp fragment (35).

Microinjection of Adenosine, DCF, and Adenosine/P1 Receptor Agonists—Microinjection was carried out via the modified method of Skoglund and Keller (37). Solutions injected were 10 mM ethylcarboxamido adenosine, 1 mM cyclopentyl adenosine, 1 mM 1, N6-ethethylcarboxamido adenosine, 1 mM Cytosine-21680 hydrochloride in MMR buffer (0.1 M NaCl, 2 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM Hepes (pH 7.5)). Approximately 100 nl of each solution was injected into lateral gastrula archenteron at stage 12. Injected embryos were incubated in 0.1 × Steinberg solution at 20 °C up to stage 35/36.

Microinjection of Morpholino Oligonucleotides (MO)—MO microinjections were performed on two-cell stage embryos immobilized in a plastic mold. The MO was diluted in MMR buffer and 5 nl of 1 mM solution was injected into each blastomere of two-cell stage embryos. After injection, the embryos were cultured in 0.1 × Steinberg solution at 20 °C up to stage 35/36. MOs designed to be antisense specific to XL_cecr1-1 or XL_cecr1–2 were purchased from GeneTools, LLC (Philomath, OR). To knock down both the XL_CECR1-1 and XL_CECR1–2 simultaneously, a mixture of both MOs was used in the experiments. The control MOs were four nucleotide-substituted versions of experimental MOs. The MO sequences containing the initiation codon were as follows (with substituted nucleotides underlined): XL_cecr1-1 MO, ATGCAGCTCATCTTTCTCAAACTCAG; 4-mis-XL_cecr1-1 MO, ATGGCAACGTATCTTAATACCATGAC; XL_cecr1–2 MO, GCAACCAATGGGAGACGCAGGCAAT; 4-mis-XL_cecr1–2 MO, GCTACTGGAGGAGGAGCAGATGAG. To confirm that the phenotype caused by the injection of the first pair of MOs is due to a specific knock down of CECR1s, we designed a second pair of MOs from a 5'-untranslated region sequence of XL_CECR1s. MOs were diluted in MMR and 5 nl of 0.1 mM solution was injected into each blastomere of two-cell stage embryos. MO sequences were as follows: Xcecr1-1–5'UTR MO, ATTCCTCTCAAGTTCTCCTCTATCAATCTCAG and Xcecr1-2–5'UTR MO, TAATTCTCTCTTCCTCTTAATATTTCTCAG.

**RESULTS**

**Identification and Characterization of X. laevis cecr1 cDNAs**—To investigate the role of the ADGF/CECR1 family in vertebrates, we attempted to isolate cDNA (s) for the *X. laevis* homologue of *CECR1*. For this, we first amplified a cDNA fragment of...
the Xenopus cecr1 homologue using degenerate primers designed according to the amino acid sequences conserved among human, pig, and zebrafish CECR1s; this was due to the lack of information regarding the nucleotide sequence of the Xenopus homologue of CECR1 on commencement of this study. We identified two cDNAs that encode relevant but distinct proteins, named Xl_CECR1-1 and Xl_CECR1-2. Comparison of the deduced amino acid sequence of Xl_CECR1-1 and -2 revealed that they shared ~40% sequence identities with Sarcophaga IDGF and human CECR1, respectively (Fig. 1A). Based on their high sequence identities (91%), Xl_CECR1-1 and -2 were considered as isoforms. The Xl_CECR1-2 nucleotide sequence was almost identical to that of a clone (accession number AY902778) deposited in the NCBI data base. Both Xl_CECR1-1 and -2 contained putative signal sequences in their N termini, suggesting that they are secretory ADGFs (51). Phylogenetic tree analysis revealed that Xl_CECR1-1 and -2 are members of the CECR1 family and that their closest relative is the chicken Gg_CECR1 (Fig. 1B). Consensus amino acids in the active site of cellular classical ADA were conserved in many CECR1s including Xl_CECR1-1 and -2 except for Val431 (Fig. 1C). We previously reported that Asp462 and His405 in Sarcophaga IDGF were essential for ADA and growth factor activity (3). These two residues are also conserved in Xl_CECR1-1 and -2 (Fig. 1A). We performed BLAST search using the Xenopus tropicalis genome data base and confirmed that the genome (scaffold 341) also encodes the orthologues for both Xl_cecr1-1 and -2, and that there is no other gene more closely related to the human CECR1 gene than the X. tropicalis gene. Thus, we concluded that the cloned cDNAs are X. laevis homologues of human CECR1.

Characterization of ADA and Growth Factor Activities of Xl_CECR1-1 and -2—We next analyzed the enzymatic properties of Xl_CECR1s and the relationship...
between their ADA and growth factor activities. For this, recombinant Xl_CECR1-1 and -2 proteins were expressed in Xenopus A6 cells, respectively. The conditioned medium containing Xl_CECR1-1 or -2 as the major protein was used for the experiments. Both Xl_CECR1-1 and -2 showed ADA activities with $K_m$ values of 260 and 230 $\mu M$, respectively, for adenosine as a substrate. The $K_m$ values of the Xl_CECR1s were in the intermediate range between those of invertebrate ($\sim$50 $\mu M$) (3) and human CECR1/ADA2 (2$\sim$200 $\mu M$) (14). The enzymatic activities of Xl_CECR1-1 and -2 were inhibited with DCF, a potent inhibitor for cellular and extracellular ADAs, but not with erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride (EHNA), a selective cellular ADA inhibitor (Fig. 2). This suggests that Xl_CECR1 has the enzymatic profile of an extracellular ADA. 2'-Deoxyadenosine was a less effective substrate than adenosine, and 5'-AMP was not a substrate for either Xl_CECR1-1 and -2, unlike in classical ADAs (Table 1) (21). These results indicate that the enzymatic activities of Xl_CECR1s share similar substrate and inhibitor specificities with human CECR1/ADA2 (52), although the $K_m$ is lower than that for human CECR1/ADA2.

Next, we examined the growth factor activities of Xl_CECR1-1 and Xl_CECR1-2 in NIH-Sape-4 cells, as these cells are sensitive to various invertebrate ADGFs (1, 3, 10, 11, 15). Although both Xl_CECR1-1 and Xl_CECR1-2 showed growth factor activity, Xl_CECR1-1 displayed activity at lower concentrations than Xl_CECR1-2 (Fig. 3), consistent with the 10 times higher specific ADA activity of Xl_CECR1-1 than Xl_CECR1-2 (data not shown). To determine the relationship between ADA and growth factor activity, we examined the effects of DCF and EHNA on this activity. The growth factor activities of both Xl_CECR1-1 and -2 were inhibited by DCF, but not by EHNA (Fig. 3). These results demonstrate that the ADA activity of Xenopus CECR1s is indispensable for growth factor activity.

**TABLE 1**

| Substrate         | Xl_CECR1-1 % | Xl_CECR1-2 % |
|-------------------|--------------|--------------|
| Adenosine         | 100          | 100          |
| 2'-Deoxyadenosine | 13           | 22           |
| 5'-AMP            | <1           | <1           |

The ADA activities of recombinant Xl_CECR1-1 and -2 were inhibited by DCF, but not by EHNA (Fig. 2). These results indicate that the enzymatic activities of Xl_CECR1s share similar substrate and inhibitor specificities with human CECR1/ADA2 (Fig. 2). This suggests that Xl_CECR1 has the enzymatic profile of an extracellular ADA. 2'-Deoxyadenosine was a less effective substrate than adenosine, and 5'-AMP was not a substrate for either Xl_CECR1-1 and -2, unlike in classical ADAs (Table 1) (21). These results indicate that the enzymatic activities of Xl_CECR1s share similar substrate and inhibitor specificities with human CECR1/ADA2 (52), although the $K_m$ is lower than that for human CECR1/ADA2.

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**FIGURE 2.** Effects of DCF or EHNA on the ADA activities of the recombinant Xl_CECR1-1 and -2. The ADA activities of recombinant Xl_CECR1-1 and -2 secreted from A6 cells were examined in the presence or absence of DCF or EHNA at the indicated concentrations, respectively. The bars indicate S.D.

**FIGURE 3.** Growth factor activities of the recombinant Xl_CECR1-1 and -2 and the effects of DCF or EHNA on their activities. Dose-response curves for stimulation of the growth of NIH-Sape-4 cells in the presence or absence of DCF or EHNA by recombinant Xl_CECR1-1 and -2. Each point represents the mean of duplicate measurements. The growth factor activity of Xl_CECR1-1 is indicated in the absence (•) or presence of DCF (grey circle) and in EHNA (○). The growth factor activity of Xl_CECR1-2 is indicated in the absence (●) or presence of DCF (grey box) and in EHNA (□).

**Analysis of the Xl_cecr1s Gene Expression during Embryogenesis**—To examine the expression of the Xl_cecr1s during embryogenesis, RT-PCR was performed using RNAs from various developmental stages with primers designed to detect both Xl_cecr1-1 and -2 mRNAs. Expression of Xl_cecr1 started at stage 20 (neurula stage), gradually increased during the neurula stage, and was sustained to stage 44 (Fig. 4). There were no differences in the expression patterns between Xl_cecr1-1 and Xl_cecr1-2 in RT-PCR using gene-specific primers (data not shown).

To determine the spatial expression pattern of the Xl_cecr1 genes, we performed whole mount in situ hybridization. The probe was a 605-bp fragment of Xl_cecr1-1 cDNA with 94% homology with the corresponding sequence of Xl_cecr1-1, which suggests cross-hybridization with the Xl_cecr1-1 transcript. The Xl_cecr1 were expressed in the somites at stage 20 (neurula stage, Fig. 5A). In addition, the cement gland was also stained at stage 22 (neurula stage, Fig. 5B). At stage 30 (tail-bud stage), the Xl_cecr1 were expressed in the somites at stage 20 (neurula stage), gradually increased during the neurula stage, and was sustained to stage 44 (Fig. 4). There were no differences in the expression patterns between Xl_cecr1-1 and Xl_cecr1-2 in RT-PCR using gene-specific primers (data not shown).

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Therefore examined the expression profiles of Xenopus homologues of an isoform of the adenosine/P1 receptor (adenosine A2A receptor, XA2A) gene. We found that Xa2a was expressed in somitic stripes and eyes in the tail-bud stage, similar to the Xl_cecr1, suggesting that Xa2a is involved in the regulation of Xl_cecr1s (Fig. 5, F and G).

Analysis of the in Vivo Functions of the Xl_CECR1s during Embryogenesis—To analyze the in vivo functions of Xl_cecr1 in embryogenesis, we used MOs to knock down Xl_cecr1. We designed two MOs specifically to target sequences surrounding the initiation codon of each of the Xl_cecr-1 and -2, and micro-injected both of them at the two-cell stage to knock down both Xl_CECR1-1 and -2. Antisense MO injection significantly reduced the amounts of both Xl_CECR1-1 and -2 as shown by immunoblotting (Fig. 6A). In contrast, there was no significant change with control MOs containing four mismatched pairs, suggesting a specific effect of these MOs. Up to the neurula stage, there were no apparent morphological differences between antisense and control MO-injected embryos, supporting our data showing that the expression of Xl_cecr1 starts at the neurula stage (Fig. 4). Developmental abnormalities were observed in MO-injected embryos at tail-bud stages. There was an intermediate phenotype with vertical or lateral bending (25%, Fig. 6, B and C), and an abnormal severe phenotype accompanied by both reduction in size and body axis bending (13%, n = 24, Fig. 6D). Conversely, embryos injected with control MO displayed no significant abnormalities (95%, n = 22, Fig. 6E). In situ hybridization experiments revealed that the expression of a gene for a basic helix loop helix transcription factor, Xmyod, was missing in some somitic stripes in MO-injected tail-bud embryos (Fig. 6, F–I), which may have contributed to the reductions in size and the abnormal body axis bending. We tested a total of 24 MO-injected embryos. Among them, 8 embryos showed a marked reduction in Xmyod expression in somites at stage 20. The phenotypes ranged from a severe one showing marked reduction in Xmyod expression in most of the somites to a mild one showing moderate reduction in Xmyod expression in a few somites. There was no tendency for the abnormal phenotype to appear in a specific region of the somite. The abnormal phenotype showing reduction in Xmyod expression was not detected in MO-injected embryos of stage 15 (data not shown), in which cecr1 mRNA had not yet been expressed, and was consistent with our notion that the phenotype is due to the knocking down of CECR1. To further confirm that the bending and truncated phenotypes observed in embryos injected with the first pair of MOs are due to the knocking down of the CECR1. As a result, similar phenotypes were observed as in the case of the first pair of MOs, and all of the MO-injected embryos showed bending and/or truncated phenotypes (n = 14, data not shown).

Analysis of Developmental Marker Gene Expression in MO-injected Embryos—We next examined whether the expression of typical developmental marker genes were affected in the MO-injected embryos (Fig. 7). Of the somite marker genes (Xmyf-5, Xmyod, cardiac actin, and six1), the gene expression of Xmyf-5 (a marker of the most dorsal and ventral tips of the somites) was significantly increased (over 16-fold compared with controls), suggesting that Xl_cecr1s are involved in sup-

stage), the Xl_cecr1s were clearly expressed in the somites, pronephros, eye, cement gland, neural tube, and neural floor plate (Fig. 5C). At stage 36, the expression patterns were similar to those of the tail-bud stage (stage 30) (Fig. 5D). Expression in the pronephros was maintained at stage 39, but expression in the other tissues diminished (data not shown). As ADA activity is indispensable for the growth factor activity of IDGF (3, 15), we assumed that Xl_CECR1s played a role in embryogenesis through adenosine/P1 receptor(s) via regulation of extracellular adenosine levels. We
pressing Xmyf-5 expression. Although Xmyod gene expression in somitic stripes was ablated partially by MOs (Fig. 6, F and H), total expression levels were not affected. We also examined markers for pronephron, eye, cement gland, and hatching gland; dramatic changes were observed in the gene expression of xsmp-30, which is a Xenopus homologue of SMP-30 (senescence marker protein-30) expressed in pronephric proximal tubules. Its expression decreased 32-fold in MO-injected embryos compared with controls, whereas there were no significant changes in the other pronephric marker genes (XNKCC2 (distal tubules), xwt1 (glomerulus), and Xlim1 (pronephros, central nervous system, and tail bud)). Of the eye marker genes, γ-crystallin, Xpitx3, and darmin-r, the gene expression of γ-crystallin decreased 16-fold in the MO-injected embryos. Expression of the marker genes for the cement gland and the hatching gland (xa and xag, respectively) was not affected significantly, consistent with the fact that the cement gland was visibly formed even in severely affected embryos (Fig. 6, B–D).

A

**FIGURE 6.** Effects of XL_cecr1 morpholino oligonucleotides on embryogenesis of X. laevis. A, immunoblotting of XL_cecr1-1 and -2 using MO-injected embryos (lane 1), 4 missed control MO (lane 2), and no treatment at stage 35/36 (lane 3). Arrows indicate the positions of XL_cecr1. B–E, effect of XL_cecr1 MOs; MOs were injected at two-cell stage for XL_cecr1-1 and -2 (B–D) or 4 missed control MO (E). Injected embryos were incubated up to stage 35/36. B and C, intermediate phenotype; bending vertically (B), or laterally (C). D, severe phenotype; both reduction and bending were observed. E, no significant phenotype obtained with 4 missed control MO. F–I, Xmyod expression at stage 20 embryos. XL_cecr1 MO-injected embryos (F and H) and no injection control (G and I). F, Xmyod expression in somitic stripes was partially ablated. Arrowheads show the ablated Xmyod expression in somitic stripes. G, the Xmyod were expressed in each somitic stripe (no injection control). H, magnified view of F. I, magnified view of G. Bars indicate 0.2 mm.

B
C
D
E
F
G
H
I

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**FIGURE 7.** Effect of XL_cecr1 morpholino oligonucleotides on the various gene expressions of X. laevis. Semi-quantitative RT-PCR was performed using primers of various developmental marker genes: MO-injected embryo (lane 1), 4 missed control MO (lane 2), and no treatment at stage 35/36 (lane 3). Relative gene expression levels were determined by RT-PCR. Amplification of ornithine decarboxylase was used to normalize the cDNA content of the samples. Xmyf-5, Xmyod, cardiac actin, and six1 are marker genes of somites. Xsmp-30, XNKCC2, xwt1, and Xlim1 are marker genes of pronephros. γ-Crystallin, Xpitx3, and darmin-r are marker genes for the eye. xa and xag are marker genes for the cement gland. Xa2a is a gene of the Xenopus homologue of the adenosine A2A receptor.
sion diminished the most in the tips of the somites (Fig. 8C). Instead, the Xmyf-5 expression was markedly up-regulated in the tail part (Fig. 8C), which may have contributed to the enhancement of the overall amount of Xmyf-5 expression detected in RT-PCR analysis (Fig. 7).

Analysis of the Mechanism of Action of XI_CECR1s and XA2A in Xenopus Embryogenesis—Next, to determine whether the adenosine/P1 receptor influences XI_CECR1 activity, we examined the effects of adenosine, an ADA inhibitor (DCF) and adenosine/P1 receptor agonists (CGS21680, 5’-N-ethylcarboxamido adenosine, or cyclopentyl adenosine) on embryogenesis by injecting them into late gastrula stage 12 embryo archenteron. Injected embryos were incubated up to stage 35/36 and their morphologies were examined. DCF caused an abnormal phenotype accompanied by reductions in both size and body axis bending, suggesting that the ADA activity of XI_CECR1s modulates their activity (Fig. 9A). Injection of adenosine, or adenosine agonists also caused abnormal phenotypes, similar to DCF, characterized by reduction in size and abnormal body axis bending (Fig. 9, B–E). We also examined the effect of the adenosine/P1 receptor agonists on the expression of the somite marker gene, Xmyod, at stage 20. In situ hybridization revealed that the Xmyod expression in somitic stripes decreased in embryos injected with either the adenosine receptor agonist, CGS-21680 hydrochloride (Fig. 9G) or cyclopentyl adenosine (data not shown). These phenotypes were similar to those obtained with the injection of cecr1 MOs, which is characterized by the ablated Xmyod expression in somitic stripes (Fig. 6, F and H). These results are consistent with our notion that XI_cecr1s function in Xenopus embryogenesis via the adenosine/P1 receptor by regulating extracellular adenosine levels.

DISCUSSION

In the current study, we first identified two Xenopus CECR1 homologues and showed that they catalyze adenosine deamination with $K_m$ values (260 µM and 230 µM) 10 times lower than those of human CECR1/ADA2 ($K_m = 2.53$ µM). Thus there might be altered levels of enzymatic activity, possibly associated with the molecular evolution from amphibian to mammal. It is noteworthy that the ADA activity of XI_CECR1s was also linked to growth factor activity, as shown for invertebrate ADGFs (3, 9–13). The differences in the specific growth factor activities between XI_CECR1-1 and -2 may reflect differences in their specific ADA activities. Thus, it is plausible that XI_CECR1s play some role in growth regulation during development via extracellular adenosine deamination.

The two Xenopus cecr1 isoforms showed similar expression profiles during embryogenesis, suggesting that they have cooperative roles. The most frequent phenotypes in the knockdown experiments were a reduction in size and abnormal bending of the body axis, which might be explained by selective changes of cell populations caused by the lack of growth factors. Given the early expression in somites of these growth factors, these results suggest that Xenopus cecr1s are involved in somite formation and differentiation during embryogenesis. This was supported by the significant increase in the amount of Xmyf-5 mRNA and the ablated expression of Xmyod mRNA in the MO-injected embryos.

The initial activation of muscle-specific genes is observed during gastrulation and concerns the two myogenic regulatory factors, XMyf-5 and XMyoD, whose transcripts are detected at stages 9.5 and 10.5, respectively. This is quickly followed by the accumulation of transcripts of cardiac actin, the first muscle structural gene expressed in vertebrates (54). Expression of the XI_cecr1 started at stage 20 (Fig. 4), and local elevation of adenosine in MO-injected embryos might have disturbed the function of Xmyf-5 and Xmyod present in somites thorough adenosine/P1 receptor signaling, resulting in developmental defects in the somites. As the gene expression of the XI_cecr1s and the adenosine/P1 receptor (Xa2a) overlaps in...
types resulting from Adgf-a (a CECR1 family in invertebrates) deficiency in Drosophila are partially explained by adenosine/P1 receptor signaling and elevations of extracellular adenosine levels (22). Similarly, adenosine concentrations could also be up-regulated in MO-injected embryos as in the Drosophila mutants (22).

We found a dramatic decrease in the number of xsmp-30 transcripts in the MO-injected embryos. Other marker genes, including that for pronephric distal tubules, XNKCC2, were unaffected in the same embryo, indicating that pronephric proximal tubules specifically failed to fully develop in the Xenopus CECR1s-deprived embryos. It was recently reported that SMP-30 acts as a survival factor in hepatocytes by regulating Akt activity (56). It might also be possible for SMP-30 to function as a survival factor during pronephros development via Akt, with expression regulated by X1_CECR1s.

Overexpression of human CECR1 is suggested to be responsible for some of the features of cat eye syndrome, such as congenital ocular coloboma and kidney defects (17, 20). In contrast, as shown in our experiments, knockdown of Xenopus CECR1s resulted in a reduction in size and abnormal bending of the body axis, suggesting an important role of Xenopus CECR1 in normal development. Thus, expression of the CECR1 should be tightly regulated, possibly to maintain the appropriate adenosine levels required for normal development.

What is the physiological function of extracellular adenosine in Xenopus embryogenesis? Our results suggest that an optimum level of extracellular adenosine is needed for normal development. ATP and the reaction products (ADP/AMP) are known to be enzymatically hydrolyzed by ectonucleotidases in the extracellular space, and the physiological significance of this has been previously discussed (55, 57). Interestingly, circular-regulated 5'-AMP seems to be the key signal that mediates murine procolipase expression in peripheral organs and induces torpor, a hibernation-like state (58). In addition, a certain level of extracellular adenosine functions as a synaptic, angiogenic, and vasculogenic modulator (55, 59–61). Thus, extracellular levels of adenosine or purine nucleotides are regulated both seasonally and developmentally. Our findings stress the important role of a novel group of extracellular ADAs linked with growth factor activity in the local metabolism of extracellular adenosine, which act via adenosine/P1 receptor-mediated intracellular signaling during embryogenesis.

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