The Signaling Protein CD38 Is Essential for Early Embryonic Development

CD38 is a multifunctional protein possessing ADP-ribosyl cyclase activity responsible for both the synthesis and the degradation of several Ca²⁺-mobilizing second messengers. Although a variety of functions have been ascribed to CD38, such as immune responses, insulin secretion, and social behavior in adults, nothing is known of its role during embryonic development. Here, we report the identification and functional expression of CD38 in Xenopus laevis, a key model organism for the study of vertebrate development. We show that CD38 expression and endogenous ADP-ribosyl cyclase activity are developmentally regulated during cellular differentiation. Chemical or molecular inhibition of CD38 abolished ADP-ribosyl cyclase activity and disrupted elongation of the anterior-posterior axis and differentiation of skeletal muscle, culminating in embryonic death. Our data uncover a previously unknown role for CD38 as an essential regulator of embryonic development.

Changes in cytosolic Ca²⁺ regulate a whole host of cellular processes including differentiation, proliferation, and muscle contraction (1). Ca²⁺ signals derive both from the extracellular space, via opening of influx channels, and from intracellular stores, through opening of Ca²⁺ release channels (1). The latter process is mediated by Ca²⁺-mobilizing messengers, including inositol triphosphate (IP₃), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP). IP₃ is the most ubiquitous of these, activating IP₃-sensitive Ca²⁺ channels located on the endoplasmic reticulum (1). cADPR targets ryanodine receptors (2), also on the endoplasmic reticulum, whereas NAADP targets two-pore channels located on acidic Ca²⁺ stores (3). Remarkably, both cADPR and NAADP are synthesized and degraded by the same family of enzymes, known as ADP-ribosyl cyclases (4, 5). The impressive catalytic portfolio of these enzymes also includes direct hydrolysis of NAD to ADP-ribose (6) and novel reactions that produce adenosine homodinucleotides from cADPR (7). Because all products produced by ADP-ribosyl cyclases regulate Ca²⁺ fluxes, these multifunctional enzymes play key roles in Ca²⁺-dependent function.

ADP-ribosyl cyclase activity was first detected in sea urchin egg homogenates and subsequently found to be widespread in animal and plant tissues (4). Molecular characterization is limited to just a few organisms, and it is remarkable that ADP-ribosyl cyclases show only modest (as little as 20%) amino acid sequence identity (8), thereby hindering bioinformatic identification of homologues. However, a core of five disulfide bonds and a catalytic glutamate residue are conserved, and their three-dimensional structures are almost identical (9, 10). These enzymes are targeted to a variety of subcellular locales including egg granules, plasma membrane, nucleus, and endoplasmic reticulum with their catalytic sites facing away from the cytosol (11). This unusual topology suggests that production of Ca²⁺-mobilizing messengers may be compartmentalized, requiring specific transporters to effect substrate import and product export (11).

In mammals, two ADP-ribosyl cyclases, BST1 and CD38, have been described at the molecular level (4, 5). Plasma membrane-targeted BST1 and CD38 function as ectoenzymes, generating messengers that are subsequently transported into the cell. They may also act as receptors that couple to several intracellular signaling pathways, further highlighting their multifunctional nature (5). Knock-out mice have been instrumental in identifying physiological roles for BST1 and CD38, with mice deficient in either isoform showing impaired immune responses (12, 13). Additionally, CD38-deficient mice demonstrate a range of defects including impaired insulin secretion (14) and social behavior (15). Moreover, CD38 has been linked to a number of human pathologies including HIV infection (16), underscoring its pathophysiological importance.

Ca²⁺ signals feature prominently throughout early development and have been shown to have highly specific roles in dorso-ventral patterning, gastrulation, and tissue differentiation (17, 18). The role of ADP-ribosyl cyclases during embryogene...
sis, however, is unexplored. Here, we use an interdisciplinary approach to reveal an essential requirement for CD38 during development of *Xenopus laevis*, a key model organism for studying vertebrate embryogenesis.

**EXPERIMENTAL PROCEDURES**

*Xenopus ADP-ribosyl Cyclases—BST1 and CD38* sequences were identified from *Xenopus* expressed sequence tag databases (www.ncbi.nlm.nih.gov). IMAGE clones (BG737274 and BX848644) were obtained (Genservice, Cambridge, UK) and fully sequenced. Protein sequences were analyzed using PSORT II, Scan-Prosite, SignalP 3.0, and BIG-PI Predictor (www.expasy.ch) and ClustalW (MacVector). *Xenopus CD38* was amplified by PCR using the primers *CD38* 1F+1R (supplemental Table S1) and inserted into the BamHI and ClaI sites of pCS2+MT, adding six Myc tags to the C terminus of CD38. A mutant lacking the antisense morpholino oligonucleotide (AMO) binding site was generated by PCR using primers *CD38* 2F+2R followed by insertion into the BamHI and ClaI sites of pCS2+. *Xenopus BST1* was amplified by PCR using primers *BST1* 2F+2R and insertion of the product into pCS2+ at the BamHI and XbaI sites. Messenger RNA was prepared using the SP6 mMESSAGE mMACHINE in vitro transcription kit (Ambion), purified on RNAeasy columns (Qiagen), and stored in RNase-free water (Sigma).

*Xenopus Embryos—In vitro* fertilized *Xenopus* embryos (19) were incubated with the indicated concentration of nicotinamide (Sigma) from stage 13 through to stage 30. 750 pg of mRNA and up to 20 ng of morpholino oligonucleotides (supplemental Table S1) were injected into each blastomere at the two-cell stage.

**Protein Analysis—In vitro translation of Xenopus ADP-ribo- syl cyclase mRNAs,** Western blot analysis of RNA-injected embryos, and enzymatic assays were all performed as described by Churamani et al. (8).

**Whole Mount Staining—In situ** hybridization was performed with digoxigenin-labeled antisense probes for *Xenopus CD38*. Immunostaining for skeletal muscle was performed with 9E10 monoclonal antibody (Insight Biotechnology) as described by Ramakrishnan et al. (20). Fluorescence images were captured using a Zeiss LSM 510 confocal microscope.

**RT-PCR—**Total RNA was purified from staged embryos using RNAeasy columns, and cDNA was synthesized using random primers and the Improm-II reverse transcription system (Promega). PCR was performed with gene-specific primers (*CD38* 3F+3R, *BST1* 3F+3R, *ODC* 1F+1R (supplemental Table S1)) and Taq polymerase (New England Biolabs), using 0.5 μl of cDNA in a final volume of 25 μl for 28 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min).

**RESULTS AND DISCUSSION**

*Xenopus* embryos are a highly tractable system for studying early vertebrate development. We therefore queried *Xenopus* expressed sequence tag databases for ADP-ribosyl cyclases and identified two clones that encoded proteins with significant amino acid identity (27–42%) to human BST1 and CD38, and that displayed marked synteny in their location in *Xenopus* and human genomes (Fig. 1A). Although homology to human ADP-ribosyl cyclases is low, there is excellent conservation of cysteines, which are involved in disulfide bond formation, as well as residues known to be important for catalysis and substrate binding (supplemental Fig. S1, supplemental Table S2). Moreover, the inferred proteins are predicted to be either a glycosylphosphatidylinositol-anchored membrane protein or a type II transmembrane protein (Fig. 1B), similar to human BST1 and CD38, respectively. Because RT-PCR failed to detect expres sion of *BST1* in *Xenopus* embryos (data not shown), we concentrated on *CD38*. Western blot analysis and whole mount immuno- staining of *Xenopus* embryos expressing *Xenopus* CD38-Myc demonstrated that it is a homodimeric glycoprotein (Fig. 1C) localized to the plasma membrane (Fig. 1D). *Xenopus* gastrulae are an ideal system for measuring recombinant ADP-ribosyl cyclase activities as endogenous activities are not detectable (8, 21, 22) (see also Fig. 2, F and G). HPLC analysis of homogenates expressing CD38-Myc revealed robust NADase activity resulting in the conversion of NAD to ADPR and weaker cyclizing (NGD to cGDPR) and base-exchange (NADP to NAADP) activities (Fig. 1E). Similar results have previously been described for mammalian CD38 (4, 5). Taken together, our results demonstrate that we have identified the *Xenopus* ortholog of CD38.

The expression pattern of *CD38* during *Xenopus* development was determined by both RT-PCR and whole mount in situ hybridization. RT-PCR analysis first detected *CD38* expression in early neurulae (stage 15), with transcript levels increasing as development progressed (Fig. 2A). Whole mount in situ hybridization with antisense probes for *Xenopus CD38* demonstrated that transcripts were initially localized to the notochord and adjacent somites (Fig. 2, B and C) but subsequently spread to the nervous system, the liver, and a region of intermediate mesoderm that might represent hematopoietic cells (Fig. 2, D and E). HPLC analysis of early gastrulae (stage 11) demonstrated a lack of endogenous ADP-ribosyl cyclase activities (Fig. 2F), consistent with our previous studies (8, 21, 22). In contrast, robust NADase activity, as well as weaker cyclizing and base-exchange activities, was detected 38 h later (stage 36) in *Xenopus* tadpoles (Fig. 2F). The relative levels of these activities were comparable with that of recombinant *Xenopus CD38* described above (Fig. 1E). Analysis of intervening stages revealed that the dominant NADase activity was first detectable at tailbud stage 28 (Fig. 2G). To determine the spatial distribution of ADP-ribosyl cyclase activity, we dissected *Xenopus* tadpoles into head, tail, and both dorsal and ventral abdominal fragments (Fig. 2H). NADase activity was strongest in the head followed by the dorsal abdomen, ventral abdomen, and tail (Fig. 2H). These results mirror the distribution of *CD38* mRNA at a similar stage (Fig. 2E). Thus, our combined analyses of both transcript and activity levels demonstrate that *CD38* is developmentally regulated in *Xenopus* embryos.

To determine the functional role of *CD38* in *Xenopus* development, we adopted a chemical-genetic approach, incubating embryos with nicotinamide, which forces ADP-ribosyl cyclase
Nicotinamide inhibited the NADase activity detectable in homogenates of *Xenopus* tadpoles in a concentration-dependent manner (Fig. 3A). Embryos incubated with nicotinamide from late gastrulae (stage 13), which is before the onset of CD38 transcription (Fig. 2A), displayed a striking reduction in the length of the anterior-posterior (head-tail) axis (Fig. 3, C and D). We also adopted a molecular approach, injecting embryos with AMOs that specifically inhibit translation of CD38 mRNA (supplemental Fig. 2, A and B). HPLC analysis of homogenates from CD38-AMO-injected embryos revealed a concentration-dependent decrease in NADase activity, with barely detectable levels in embryos injected with 20 ng of CD38-AMO (Fig. 3B). Robust NADase activity was detected in embryos injected with 20 ng of either control morpholino (Fig. 3B) or BST1-AMO (supplemental Fig. S2C). The CD38-AMO caused a reduction in the length of the anterior-posterior axis (Fig. 3, E and F), similar to that observed with nicotinamide. These tadpoles failed to swim, either spontaneously or in response to touch, and subsequently died. Defects were not discernible in embryos injected with control MO (Fig. 3C). Partial rescue of the anterior-posterior axis defect was effected by co-injecting CD38-AMO with CD38 mRNA that lacked the AMO target sequence (Fig. 3G), indicating that it was caused by specific inhibition of CD38. Our results suggest that CD38 accounts for all ADP-ribosyl cyclase activity detectable in early *Xenopus* embryos and that this activity is essential for normal development.

Finally, we examined the mechanistic basis underlying disruption of embryonic development by nicotinamide and CD38-AMO. Transcripts for CD38 are first detected in the notochord and somites of *Xenopus* neurulae (Fig. 2D), and disrupting somite development has been shown to cause reductions in anterior-posterior axis length similar to those described here (23). Because skeletal muscle is the main tissue type formed by amphibian somites, we examined its differentiation in early *Xenopus* embryos with inhibited CD38 function. Skeletal muscle was clearly visible as chevron-shaped blocks along the back of control embryos (Fig. 3, H and I). In contrast, nicotinamide-treated and CD38-AMO-injected embryos formed fewer muscle blocks than controls, and they lacked their distinctive chevron shape (Fig. 3, J and K). Skeletal muscle was only detected in the most anterior regions of the trunk. Our results demonstrate that CD38 has an essential role in the differentiation of skeletal muscle.
Using *X. laevis*, we show that CD38 is required for embryonic development and more specifically skeletal muscle differentiation. Studies on knock-out mice have failed to detect a role for CD38 in embryogenesis, which may be explained by compensatory mechanisms in mutant mice. Indeed, compensatory Ca\(^{2+}\)/H\(_{11001}\) influx maintains NAADP-dependent agonist-evoked calcium signaling in pancreatic acinar cells from *CD38*-/-/ mice (24). Our findings are consistent with *in vitro* studies showing CD38 regulates differentiation of HL60 cells (25). How CD38 contributes to muscle differentiation requires further work. In mammals, CD38 also functions as a receptor (5) and we cannot exclude a similar role in *Xenopus*. It is notable however that nicotinamide, which blocks ADP-ribosyl activity, phenocopies the *CD38* morpholino and that Ca\(^{2+}\)/H\(_{11001}\) transients regulate skeletal muscle development.

**FIGURE 2.** *Xenopus CD38* is developmentally regulated. A, RT-PCR analysis of *Xenopus CD38* expression in staged *Xenopus* embryos. Ubiquitously expressed ornithine decarboxylase (ODC) and RNA (stage 33) without reverse transcriptase (−RT) were used as controls. Embryos were collected as blastulae (stage 8), gastrulae (stages 12 and 13), neurulae (stages 15 and 22), and tailbud stages (stages 25 and 33). B–E, whole mount in situ hybridization for CD38 expression (purple stain) in *Xenopus* embryos (anterior to left and posterior to right). B, dorsal view of a stage 16 embryo. C, cross section of a stage 16 embryo with neural plate (np), notochord (n), somites (s), and endoderm (e) indicated. D, lateral view of a stage 23 embryo. E, lateral view of a stage 30 embryo. F, time courses for endogenous ADP-ribosyl cyclase activities in *Xenopus* homogenates from stage 11 and stage 36 embryos, showing production of ADPR from NAD, cGDPR from NGD, and NAADP from NADP and nicotinic acid (NA). Nic, nicotinamide. G, stage-dependent NADase activity (ADPR production from NAD) in *Xenopus* embryo homogenates. H, region-specific NADase activity in fragments from stage 36 embryos. H, head; D, dorsal abdomen; V, ventral abdomen; T, tail. Error bars represent mean ± S.E. of 36–64 embryos (***, p < 0.005).

**FIGURE 3.** Inhibition of CD38 disrupts *Xenopus* development. A and B, time courses for endogenous NADase activity (ADPR production from NAD) in *Xenopus* homogenates from stage 30 embryos. A, homogenates incubated with increasing concentrations (0, 1, 3, and 30 mM) of Nic. B, homogenates from embryos injected with either 20 ng of control morpholino (CMO) or 10–20 ng of *CD38*-AMO. C–F, lateral views of stage 30 embryos (head on the left). C, untreated control embryo. D, embryo incubated in 30 mM Nic from stage 13 to stage 30. E, embryo injected with 20 ng of CMO. F, embryos injected with 20 ng of *CD38*-AMO. G, length of anterior-posterior (head-tail) axis of *Xenopus* embryos with inhibited CD38 activity. Cont, control; Nic, 30 mM nicotinamide; CMO, 20 ng; AMO, 20 ng of *CD38*-AMO; RNA, 1.5 ng of *CD38* mRNA. Error bars represent mean ± S.E. of 36–64 embryos (***, p < 0.005). H–K, lateral views of stage 30 embryos stained for skeletal muscle with 12/101 monoclonal antibody (head on the left). H, untreated control embryo. I, embryos incubated in 30 mM Nic from stage 13 to stage 30. J, embryo injected with 20 ng of CMO. K, embryos injected with 20 ng of *CD38*-AMO.
tal muscle differentiation (26). Moreover, both ryanodine receptors and two-pore channels, targeted by cADPR and NAADP, respectively, regulate skeletal muscle differentiation (27–29). Messenger production by CD38 may therefore contribute to Ca\(^{2+}\)-dependent muscle development.

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