Anbmp2/4 is a new member of the transforming growth factor- β superfamily isolated from a crinoid and involved in regeneration

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Invertebrates have frequently been used to help understand the complexities of regulatory gene function and evolution. The bone morphogenetic proteins (BMPs) are a highly conserved group of secreted regulatory factors that play an important part in early embryonic patterning. In the present study we have used the remarkable regenerative potential of crinoid echinoderms to explore the BMPs' site of expression in an adult developmental programme. Our results suggest that a crinoid BMP2/4 homologue is actively involved during the early stages of blastemal regeneration at a time when fundamental patterns are being established. This supports the idea of an evolutionary developmental programme where essential gene families are conserved throughout phylogeny in terms of both expression and function.

Keywords: regeneration; invertebrates; bone morphogenetic proteins

1. INTRODUCTION

Bone morphogenetic proteins (BMPs) were originally identified from their role in bone formation (Wozney *et al.* 1988) but are now recognized as playing an important part in the regulation of many fundamental developmental processes, for example neural induction in vertebrates and invertebrates (Holland & Holland 1999). BMPs are members of the transforming growth factor- β (TGF- β) superfamily, and within the BMP subfamily, further categories can be recognized, including the BMP2–4 subgroup and the BMP5–7 subgroup (Hogan 1996). The active gradient established by these secreted ligands is one of the essential factors responsible for generating the positional information that underlies developmental patterning. It is now accepted that the ways in which these gradients are typically originated involve regulation by a secreted inhibitor and/or spatially and temporally regulated ligand synthesis (Myers *et al.* 2002). Vertebrate BMP antagonists include noggin and chordin, and these act as neural inducers by operating as functional antagonists to BMP2 or BMP4. Indeed, BMPs act to maintain non-neural ectodermal identity, and the primary role for the neural inducers is to affect an area of ectoderm where the BMPs are antagonized and are allowed to adopt a neural fate (Ducy & Karsenty 2000). The BMP signalling pathway may pre-date the emergence of the bilateria; indeed, members of this regulatory network have been identified in cnidarians and the proposal made that an ancestral role in neuroepithelial

patterning underlies the highly conserved nature of this family (Samuel *et al.* 2001; Hayward *et al.* 2002).

TGF- β homologues have been identified in the coelomopore complex of sea cucumber embryos (Harada *et al.* 2002) and in embryos of the sea urchin *Strongylocentrotus purpuratus* (Stenzel *et al.* 1994). In the latter species, the *univin* gene may be critical for early developmental events, while in the later pluteus stage of *Tripneustes gratilla* (Hwang *et al.* 1999), *TgBMP2/4* is thought to have an important regulatory role in gut patterning and spicule formation. A more detailed study on patterning in the sea urchin animal–vegetal embryonic axis has recently confirmed a role for BMP2/4 in influencing the regulation of cell fate within the ectoderm (Angerer *et al.* 2000). Here, experiments have shown that sea urchin BMP2/4 and its antagonist noggin play a crucial part in regulating the position of the ectoderm–endoderm boundary as well as epidermal versus non-epidermal fate within the ectoderm (Angerer *et al.* 2000). We have recently demonstrated, using an immunochemical approach, that a TGF- β -like molecule is expressed in normal and regenerating arms of crinoids (Patruno *et al.* 2002). Here, we report the sequence of a native member of this superfamily of secreted growth factors from *Antedon bifida* (*Anbmp2/4*) and demonstrate its temporal and spatial expression pattern in regenerating arms.

2. MATERIAL AND METHODS

The crinoids (*A. bifida*) employed in this study were collected from Kristineberg Marine Research Station (Sweden). Animals were collected by means of a sediment grab and kept in a circulating seawater system at 12 °C. Experimental regeneration of

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arms was induced by mimicking the conditions of natural autotomy (distal or intermediate amputations; see Candia Carnevali *et al.* 1993; Patruno *et al.* 2002). Tissue samples were prepared from both normal intact arms and regenerating arms of at least three animals. Regeneration was monitored at fixed times: early in the regenerative period (15, 24, 48 and 72 h after amputation) and at an advanced stage (one week after amputation).

(a) *RNA isolation, cDNA synthesis and reverse transcription-PCR*

Total RNA from normal and regenerating arms of *A. bifida* was isolated using the TRIZOL reagent (Gibco, BRL) and reverse-transcribed into double-stranded cDNA by means of the rapid amplification of cDNA ends (RACE) Marathon kit (Clontech). A pair of degenerate primers were designed from a conserved amino acid sequence of TGF- β molecules from mammalian and invertebrate species and used in an initial PCR step (forward, 5'-ACTCTAGATGGATYRTNGCICC-3'; reverse, 5'-ACGAATTCCTTANCGRCANCCICA-3'; modified from Stenzel *et al.* 1994). A second PCR step using nested primers was necessary to amplify a 150 bp product (forward, 5'-ACTCTAGAGCTTAYTWYTGYSAGG-3'; reverse, 5'-ACGAATTCGTNGGNACRCARCA-3'). The PCR reaction (10 mmol of each primer, 10 $\times$ PCR buffer, pH 8.7, 2 mM of each dNTP mix, 3.5 mM MgCl₂ and 2.5 units of *Pfx* Taq DNA polymerase; Gibco, BRL) was carried out under the following conditions: one cycle of 94 °C for 1 min; 45 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 3 min, and a final incubation at 72 °C for 10 min. The second PCR was performed to amplify 1 μ l of the primary PCR product under the same conditions but using a temperature of 50 °C instead of 55 °C. The 150 bp product was cleaned up by a phenol-chloroform-isoamylalcohol extraction, blunt ended with a Klenow fragment and cloned with 5DH α competent cells (into a *Sma*I-digested pBluscript II SK (-) vector (Stratagene)). Subsequently, using a specific forward primer based on the known 150 bp sequence, called 3'TNH (5'-CGACAAATCACGCGATTGTACAAACTCTAG-3'), and a specific adaptor primer linked to the 3' end of the cDNA (Marathon kit, Clontech), a full-length copy of the gene fragment of interest (707 bp including the 3' untranslated region of the *AnBMP2/4* clone; GenBank accession no. AF452464) was obtained. Sequencing was carried out using the ABI Prism 377 DNA sequencer and the ABI Prism BigDye sequencing kit (PE Biosystems). Reverse transcription-PCR (RT-PCR) was carried out on cDNAs obtained from non-regenerating and regenerating arms together with tissues from the oral part of *A. bifida* using one forward primer from the 3' coding region, TNH-F, 5'-GACAAATCACGCGATTGTAC-3', and one reverse primer from the 3' non-coding region, AB320, 5'-GCAAAGCAATGAATTTACACC-3'. Amino acid sequence alignments and the molecular phylogenetic relationships within the BMP family of proteins were generated using the neighbour-joining (Saitou & Nei 1987) and the quartet puzzling maximum-likelihood methods.

(b) *PCR on genomic DNA*

Antedon bifida genomic DNA was extracted using a salting-out protocol from 200–300 mg of crinoid tissue (Patwary *et al.* 1994). We amplified 200 ng of extracted DNA by PCR using TNH-F and AB320 primers.

(c) *Whole-mount in situ hybridization*

Linearized DNA obtained as described in § 2a (*ca.* 700 bp) was used as a template to generate sense and anti-sense RNA probes by using bacteriophage SP6 or T7 RNA polymerases according to a protocol from Nieto *et al.* (1996). The procedures for fixation and hybridization of *A. bifida* arms were carried out according to Holland *et al.* (1996). Differently from these protocols, crinoid tissue was bleached with 6% hydrogen peroxide phosphate-buffered Tween20 (PBT) for 30 min and washed in PBT before the hybridization step. Control experiments refer to samples hybridized with a sense RNA probe. Transcripts were identified by using the DIG nucleic-acid detection kit (Roche). The hybridized specimens were viewed using a stereomicroscope, connected to a camera (Olympus). For histological examination, hybridized whole-mount samples were post-embedded in Epon-Araldite and thick (10 μ m) and semithin serial sections (*ca.* 1 μ m, counterstained with toluidine blue) were obtained with the LKB Ultratome V microtome.

3. RESULTS

(a) *Isolation of an Antedon bifida BMP2/4*

Theoretical translation of the 3'RACE-PCR product revealed a strong similarity to sequences of other BMPs (figure 1). The highest degree of amino acid identity (82%) was evident with the sea urchin *T. gratilla* BMP2/4 and other known sea urchin BMP2/4 homologues (81% with *S. purpuratus* and *Loligo vulgaris* (data not shown)), whereas it was slightly lower (75%) with the asteroid *Asterias rubens*. The *Drosophila* BMP2/4 homologue, DPP, also showed a high amino acid identity with *AnBMP2/4* (77%) as well as with human BMP-2 (81%) and BMP-4 (78%). By contrast, 60A, another *Drosophila* TGF- β homologue, and *univin* from the sea urchin *S. purpuratus* both gave low scores when aligned with *AnBMP2/4* (55% and 67%, respectively).

The phylogenetic tree shown in figure 2 confirmed the position of *AnBMP2/4* in the BMP2–4 TGF- β subfamily. Furthermore, the *A. bifida* sequence was more closely related to *Drosophila* DPP than to the *Drosophila* protein 60A.

(b) *Expression of Anbmp2/4 in Antedon bifida during regeneration of the arm*

Figure 3 illustrates the main anatomical features of a regenerating arm at one week after amputation. Cellular expression of *Anbmp2/4* monitored by *in situ* hybridization carried out on non-regenerating and regenerating arms of *A. bifida* took place relatively rapidly following amputation. The first hybridization signal was detected at the beginning of the typical repair phase (*ca.* 24 h after amputation) along the ambulacral-groove side of the regenerating arm (figure 4a). Resin sections of these hybridized arms showed an even expression along the coelomic canal epithelium (figure 4b) but not in the brachial nerve (figure 4c). The hybridization signal was not observed in the non-regenerating arm or less than 24 h after amputation probably owing to the low level of mRNA. However, by means of a more sensitive approach, such as RT-PCR, we were also able to detect *Anbmp2/4*

Figure 1. Alignment of vertebrate and invertebrate BMP2/4 homologues and the gene portion of *Antedon bifida* (top row) using CLUSTALW. Dots within the sequences and asterisks in the bottom line indicate amino acid identity. Dots in the bottom line indicate amino acid similarities. GenBank accession numbers are as follows: (2) *Asterias rubens* BMP2/4, CAB63584; (3) *Triptoneustes gratilla* TGBMP2/4, AF133305; (4) *Strongylocentrotus purpuratus* BMP2/4, AF119713; (5) *Homo sapiens* BMP2; (6) *H. sapiens* BMP4, BAA06410; (7) *Danio rerio* BMP2/4, AAC60286; (8) *Junonia coenia* DPP, AAB46367; (9) *S. purpuratus*–UNIVIN, P48970.

Figure 2. Molecular phylogenetic relationship of *AnBMP2/4* to the TGF- β family of proteins estimated by the neighbour-joining method (Saitou & Nei 1987), which shows the relationship between *AnBMP2/4* and both vertebrate and invertebrate sequences. Echinoderm sequences are from sea urchins *S. purpuratus univin* and *SpBMP2/4*, *Lytechinus variegatus BMP2/4* and *T. gratilla BMP2/4*, the starfish *A. rubens BMP2/4* and the sea cucumber *S. japonicus BMP2/4* (GenBank accession no. 27530619). Other sequences are human BMP2 (NP_001191) and BMP4 (GenBank accession no. 1311542), the mollusc *Patella vulgata BMP2/4* (GenBank accession no. 20975762) and the cnidarian *Acropora millepora DPP* (GenBank accession no. 21449273). Branch lengths are proportional to evolutionary distance corrected for multiple substitutions with the scale denoting 0.1 amino acid substitutions per site (MEGA2 program). The numbers indicate the strength of each node as assessed by bootstrap analysis (500 replications). Only the most significant numbers indicating the strength of each node as assessed by bootstrap analysis (500 replications) are shown.

mRNA in non-regenerating arms (figure 5). PCR on genomic DNA, using the same primers as for RT-PCR, did not reveal specific bands probably because of the presence of a long intron sequence (refer to the human genome for BMP2 and BMP4 at NCBI-MapViewer). Expression remained high and spatially restricted to the coelomic epithelium for the overall repair phase (24–48 h after amputation). At early regenerative stages (48–72 h after amputation), the regenerating arm presents a clearly recognizable regenerating bud (blastema; figure 6b), which is positively stained. As in the previous stages, the expression can also be seen at the level of the ambulacral region (figure 6a), whereas the brachial nerve is not involved (figure 6a). At these same stages, a marked *Anbmp2/4* expression was localized at the level of the coelomic-canal epithelium, particularly adjacent to the

amputation site (figure 6c) and in the blastema (figure 6e,f).

At the advanced stage of one week after amputation, when the regenerating arm becomes anatomically well defined, the pattern of expression appeared to change. Both whole mounts and resin sections revealed specific staining localized in the proximal portion of the regenerate (figure 7) where differentiation of new tissues (in particular elements of the skeletal tissue) is in progress (figure 7*d*). No signal was detectable in the brachial nerve of the same samples (figure 7*b*). In contrast to the early stages of regeneration, the signal in the coelomic epithelium decreased (figure 7*b*). Semithin toluidine-blue-stained sections confirmed that in the regenerate the hybridization signal was restricted to blastemal cells (figure 7*d*). At two weeks after amputation the signal was no longer detectable

Figure 3. (a) Schematic reconstruction of a regenerating arm at one week after amputation in sagittal section. (b) Inset from (a) showing details of the basiepithelial nerve plexuses at the level of the ambulacral epithelium and the coelomic lining. Abbreviations: Abp, ambulacral basiepithelial plexus; bn, brachial nerve; cbp, coelomic basiepithelial plexus; cc, coelomic canals; R, regenerating arm; o, ossicle. (After Candia Carnevali *et al.* 1993.)

Figure 4. (a) Whole-mount *in situ* hybridization using the *Anbmp2/4* probe at 15 h (bottom) and 24 h (top) after arm amputation in *Antedon bifida*. A high level of transcription all along the oral side of the regenerating arm (arrowhead) was observed; the arrow indicates the presumed site of future blastema formation (scale bar, 1 mm). (b, c) Sections of the sample shown in (a) (resin thick sagittal section, 24 h after amputation). (b) Expression was clearly seen along the coelomic epithelium (arrow), but not (c) in the brachial nerve (bn). The arrowhead in (b) indicates the ambulacral groove. Scale bars, (b) 100 μ m; (c) 70 μ m.

by *in situ* hybridization in any tissues of the regenerating arm (data not shown).

4. DISCUSSION

(a) *AnBMP2/4* is a homologue of vertebrate *BMP2/4*

In this study, a molecular approach has been used to elucidate the nature of TGF- β -like proteins in crinoids. Phylogenetic analysis of *AnBMP2/4* confirmed the evolutionary position of this new gene fragment in the TGF- β superfamily. It has been named *Anbmp2/4* as it appears to be closely related to the BMPs. The peptide sequence deduced from translating this region of the gene fragment contains 76 amino acids that show high homology with

Figure 5. RT-PCR on cDNAs prepared from different tissues of *Antedon bifida*. Primers (TNH-F and AB320) were designed from the *A. bifida* sequence corresponding to the 3' non-coding region: lane 1, 100 bp ladder molecular weight marker (Gibco, BRL); lane 2, normal non-regenerating adult arm; lane 3, tegmen from 'mouth' soft tissue; lanes 4 and 5, regenerating arms at 24 h and one week after amputation, respectively.

those at the C-terminal end of proteins belonging to the TGF- β superfamily of secreted growth factors. According to sequence similarity, vertebrate BMPs are subdivided into two classes (Kingsley 1994). One includes BMP-2 and BMP-4, which are related to *Drosophila* DPP. The other is the 60A subclass, which is related to *Drosophila* 60A. Our data indicate that *AnBMP2/4* is a member of the DPP subfamily rather than a member of the 60A subfamily (figure 2). Apart from *univin*, four other BMP2/4 homologues have been sequenced in echinoderms: two from the echinoids *T. gratilla* (Hwang *et al.* 1999) and *S. purpuratus* (Angerer *et al.* 2000), one from the asteroid *A. rubens* (Lelong *et al.* 2001) and one from the holothuroid *Stichopus japonicus* (Harada *et al.* 2002). Another BMP subfamily gene has been sequenced in *S. purpuratus* (Ponce *et al.* 1999) and is related to the BMP5-7 subfamily. Ponce *et al.* suggest that only a few invertebrate deuterostome TGF- β molecules have been isolated so far because most data are based on cDNAs from a restricted number of embryonic stages. Indeed, echinoderm postembryonic juvenile and adult phases are produced by very different developmental mechanisms and genes other than those used for embryogenesis might well be employed. Moreover, there might be the possibility of

Figure 6. Whole-mount (a,b) *in situ* hybridization using the *Anbmp2/4* probe in *Antedon bifida*. (a) Frontal view of a regenerating arm at 48 h after amputation. The brachial nerve does not express *Anbmp2/4* (arrow), while the ambulacral region of the regenerating arm was intensively stained (arrowhead); scale bar, 1 mm. (b) Lateral view of a regenerating arm at 72 h after amputation. The arrow indicates the regenerating blastema; scale bar, 1 mm. (c) Thick resin sagittal section of (a), showing a widespread staining along the coelomic epithelium (arrow), especially close to the regenerating arm tip (arrowhead); scale bar, 60 μ m. (d) Thick resin sagittal section of a control sample. No staining was observed in the tissues of the regenerating arm; scale bar, 60 μ m. (e,f) Thick resin sagittal sections of (b) revealed a specific strong staining in the proximal region of the regenerating blastema (arrowheads); scale bars, 60 μ m and 30 μ m, respectively. Abbreviations: bn, brachial nerve; cc, coelomic canals; o, ossicles.

another BMP homologue in *A. bifida* because our group found two isoforms of a TGF- β -like molecule during regeneration using an electrophoretic approach (Patruno *et al.* 2002).

(b) Expression pattern of AnBMP2/4 in regenerating crinoids

Here we describe, for the first time, to our knowledge, the presence and putative role of an orthologous crinoid BMP gene during the arm regenerating process in an adult echinoderm. It is important to note that there have been few studies regarding specification of cells in adult echinoderms. We know from other studies conducted on 'classic' animal models that BMP signalling pathways are involved

Figure 7. Whole-mount *in situ* hybridization using the *Anbmp2/4* probe at one week after amputation in *Antedon bifida*. (a) The regenerate strongly expresses *Anbmp2/4* (arrow); scale bar, 1 mm. (b,c) Thick resin sections of (a) show a localized staining in the regenerating arm; (b) scale bar, 200 μ m; (c) scale bar, 100 μ m. (d) Serial semithin section of (a) stained with toluidine blue reveals that the staining involves the blastemal cells at the proximal part of the regenerate; scale bar, 50 μ m. In (c) and (d), circles indicate the sites of the proximal regenerate where new calcite spicules are formed. (e) Thick resin section of control; scale bar, 100 μ m. (f) Semithin control stained with toluidine blue; scale bar, 200 μ m. Abbreviations: o, ossicle; r, regenerate; bn, brachial nerve; cc, coelomic canals.

in the regulation of axis formation and neural–non-neural boundaries (De Robertis & Sasai 1996; Chitnis 1999). In vertebrates and in *Drosophila*, similar functions of BMP4 and DPP confirm that a comparable molecular mechanism for distinguishing non-neural ectoderm from neural ectoderm was probably present in the common ancestor of all bilaterally symmetrical animals (Holland & Holland 1999). For instance in amphioxus, an animal widely accepted as representing ancestral chordates, a BMP homologue, *AmphiBMP2/4*, is downregulated in the presumptive neural plate (Panopoulou *et al.* 1998) and in ascidians, *HrBMPb* functions as a neural inhibitor and epidermal inducer (Miya *et al.* 1997). *In situ* hybridization and mRNA microinjection in *S. purpuratus* confirmed that BMP signalling might be considered a vertebrate homologous developmental coordinate system (Angerer & Angerer 2000; Angerer *et al.* 2000). However, the functional

role of BMPs during growth in adult echinoderms is still unknown. The blastemal regeneration that occurs in crinoids is a novel model for studying the putative role of BMPs. Normal arm growth in crinoids is characterized by a phenomenon of protein turnover that is enhanced during blastemal regeneration (Patruno *et al.* 2001). In the latter process, migratory cells from both the coelomic canals and the brachial nerve show the characteristic features of undifferentiated stem cells (Candia Carnevali & Bonasoro 2001).

It is not surprising, therefore, that a BMP gene is active during normal arm growth (detected by RT-PCR but not by *in situ* hybridization, probably because of a low level of mRNA) and may increase its expression during regeneration. *In situ* experiments revealed that the studied gene is not expressed in the brachial nerve. This could be seen as comparable to the situation described in ascidians (Miya *et al.* 1997) or in developing sea urchin larvae where the *SpBMP2/4* regulates the position of the ectoderm–endoderm boundary (Angerer *et al.* 2000). In this study, we have shown that, shortly after arm amputation, the coelomic epithelium of *A. bifida* expresses a marked amount of *AnBMP2/4* transcript. It is known from previous studies (Candia Carnevali & Bonasoro 2001) that cells from the coelomic canals migrate towards the arm tip to become blastemal cells. These cells showed strong localization of *Anbmp2/4* up to one week after amputation, mainly in the proximal region of the regenerate. Then, the signal faded during more advanced phases of regeneration. One might speculate here that *Anbmp2/4* could be involved in the specification of migratory stem cells derived from the coelomic epithelium. The latter in echinoderms is of mesodermal (mesenchymal) origin and as such shows a clear lineage linkage (at least in echinoids) to the so-called set-aside cells of the embryo and larva (Arenas-Mena *et al.* 2000). These are thought to be pluripotent or totipotent cells ‘set aside’ during development and used in building the adult body plan. They are manifest in adults as coelomic epithelial cells or the coelomocytes that arise by delamination from the coelomic epithelium.

From one week after amputation the new arm presented signs of differentiation with particular reference to the process of skeletogenesis evident in the formation of several new calcite elements in the proximal part of the regenerate. At this stage, *Anbmp2/4* is still expressed widely in the regenerate where the new skeleton is growing rapidly and new muscle, ligaments and nerve cells are being formed.

These results, together with our previous findings (Patruno *et al.* 2002), support the idea of an evolutionary developmental programme where essential gene families are conserved in terms of expression and are necessary to establish general body patterns in the embryo as well as in the adult.

We thank all the staff at Kristineberg Marine Research Station and the EU TMR ‘ARI’ programme for support. Thanks also to Professor G. Wray (Duke University), all his laboratory and Dr Maccatrozzo for continuing scientific advice; an Anglo-Italian/British Council-MURST exchange grant; the University of London Central Research Fund; the Royal Holloway Research Strategy Fund; a Thomas Holloway Studentship (M.P.); the Italian COFIN 2000 MURST Project; the Italian CNR-Biotechnology Research Project, Subproject Environ-

mental Biotechnology; a NATO Scientific Investigations grant; and Lalla.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.