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Original Article

Cloning of the *Xenopus* integrin α_V subunit and analysis of its distribution during early development

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ABSTRACT One striking feature of the integrin α_V subunit is its ability to associate with at least five different β subunits (β_1, β_3, β_5, β_6 and β_8) to form functional receptors. These receptors are involved in diverse biological processes, such as differentiation, cell adhesion and migration. Here we report the cloning of the *Xenopus* homolog of the integrin α_V subunit. Integrin α_V mRNA and protein are maternally supplied and present throughout development. During gastrulation and neurulation α_V protein appears on cell membranes of all three germ layers. In tailbud stage embryos great amounts of the α_V protein can be observed in the inner layer of the ectoderm and in the endothelial cells lining the pharynx and gut.

KEY WORDS: *Xenopus, integrin, α_V, development*

Introduction

Integrins are heterodimeric transmembrane glycoproteins composed of non-covalently bound α and β subunits. They function as receptors which mediate cell matrix and cell cell adhesion by binding to extracellular matrix proteins or cell surface molecules of the immunoglobulin superfamily. The extracellular domains of both integrin subunits are involved in ligand recognition and binding (Hynes, 1992). The cytoplasmic domain of the α subunit is thought to modulate receptor ligand interaction (O'Toole *et al.*, 1994) while the intracellular domain of the β subunit interacts with cytoskeletal components such as α-actinin (Otey *et al.*, 1993) or talin (Horwitz *et al.*, 1986) and with regulatory and signal transducing molecules such as focal adhesion kinase (FAK, Schaller *et al.*, 1995). Thus, integrins form a structural link between the extracellular environment and the cytoskeleton and participate in signal transduction across the plasma membrane (Dedhar and Hannigan, 1996; Schwartz *et al.*, 1995). Integrin dependent adhesion can alter normal cell function and activate various signal transduction pathways thereby regulating cell growth, differentiation and gene expression (Adams and Watt, 1993; Lallier *et al.*, 1994; Clark and Brugge, 1995). So far, fifteen different α subunits and eight different β subunits have been characterized, which, in various combinations, are able to form at least 21 different functional integrins (Hynes, 1992).

While most of the α subunits form heterodimers with only one particular β subunit (such as α_1 and α_5, which only pair with β_1), a number of others can assemble with two different β subunits (e.g., α_4 with β_1 and β_7 and α_6 with β_1 and β_4). The α_V subunit is exceptional in that it is able to associate with at least five different β subunits (β_1, β_3, β_5, β_6 and β_8). Most of the α_V receptors recognize either fibronectin ($\alpha_V\beta_6$; Busk *et al.*, 1992) or vitronectin ($\alpha_V\beta_5$; Smith *et al.*, 1990) or both ($\alpha_V\beta_1$; Bodary and McLean, 1990; Vogel *et al.*, 1990). The $\alpha_V\beta_3$ integrin, however, also binds thrombospondin, von Willebrand factor, osteopontin and bone sialoprotein 1 (Gladson and Cheresh, 1994). This ability to form functional heterodimers with various β subunits and thus recognize different ligands enables α_V integrins to participate in a variety of biological processes including retinal outgrowth (Neugebauer *et al.*, 1991), bone resorption (Davies *et al.*, 1989), differentiation (Adams and Watt, 1991), angiogenesis (Brooks *et al.*, 1994), metanephrogenesis (Wada *et al.*, 1996), phagocytosis (Blystone *et al.*, 1994), metastasis proliferation (Seftor *et al.*, 1992) and immune response (Savill *et al.*, 1990).

Analysis of α_V integrin function during embryogenesis has been performed in chick, in mouse and in amphibians. In the chick retina α_V is involved in neuronal outgrowth on vitronectin. The attachment and outgrowth of retinal neurons is integrin dependent and developmentally regulated (Neugebauer *et al.*, 1991; Reichardt and Tomaselli, 1991). Morphogenesis of the mouse kidney was disturbed by using either α_V specific antisense oligonucleotides or α_V specific antibodies in a metanephric organ culture system (Wada *et al.*, 1996). In *Pleurodeles*, α_V mRNA and protein are broadly expressed in eggs and early embryos. During gastrulation the α_V protein is mainly found on migrating mesodermal cells (Alfandari *et al.*, 1995). Ramos *et al.*, (1996) demonstrated the presence of α_V on cell surfaces of *Xenopus* blastula and gastrula stage embryos. The authors assume that α_V integrins are involved in integrin fibronectin interactions during *Xenopus* gastrulation.

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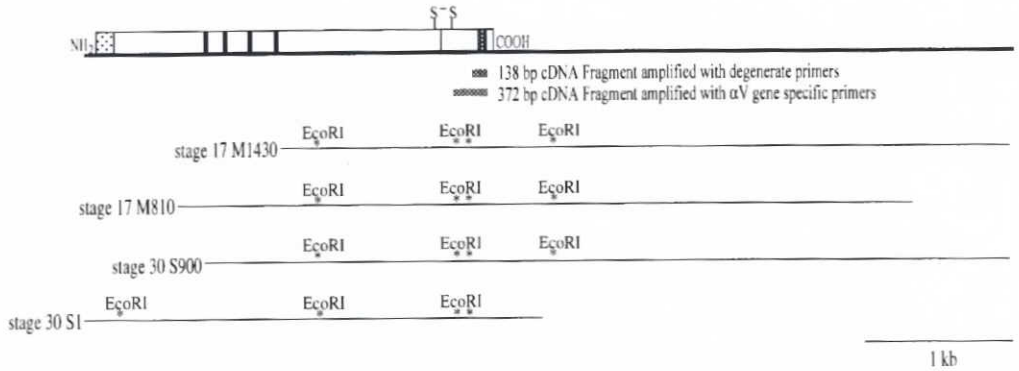

Fig. 1. Cloning of the Xenopus integrin α_V subunit. *The α_V cDNA reconstructed from overlapping λ clones comprises 6097bp, drawn as a bold line. The box indicates an open reading frame with a putative signal peptide (stippled box), a long extracellular domain with four metal binding sites (black bars), a single transmembrane domain (black box with white stipples) and a short cytoplasmic segment. The post-translational cleavage site (vertical line) and the disulfide bond (S-S) are shown. The PCR generated fragments which were used to isolate the α_V cDNA are indicated by stippled bars. Four cDNA clones were isolated from a Xenopus neurula stage (s17M) and a Xenopus tailbud stage (s30S) library. Stars represent EcoRI/restriction sites. The clone s30S1 contains the full-length open reading frame of the Xenopus α_V subunit.*

We have previously analyzed the distribution of the $\alpha_5\beta_1$ fibronectin receptor during the early development of *Xenopus laevis* (Joos *et al.*, 1995). Here we report the cloning of the *Xenopus laevis* homolog of the integrin α_V subunit, which, in combination with different β subunits, may form further fibronectin receptors functioning in early development. We show that α_V mRNA and protein is present throughout development. During early development α_V protein was found on all cells of all three germ layers. Prominent α_V protein could be observed in late tadpole stage embryos in the sensorial layer of the ectoderm and in the endothelial cells lining the pharynx and the gut.

Results

Cloning and sequence analysis of the Xenopus integrin α_V cDNA

A *Xenopus* integrin α_V cDNA fragment was obtained by PCR amplification using the same strategy as described in Joos *et al.*, (1995). Degenerate oligodeoxynucleotide primers corresponding to the highly conserved G-F-F-K/R-R motif of the cytoplasmic domain were used together with a λ arm specific primer to amplify a 138bp integrin α_V cDNA fragment from a λgt10 *Xenopus* neurula stage cDNA library (Kintner and Melton, 1987). Based on this sequence an α_V gene specific oligodeoxynucleotide primer was synthesized. This primer was used together with the same λ arm specific primer in an additional PCR, which was now performed under more stringent conditions. A 372bp integrin α_V cDNA fragment was obtained (Fig. 1). This partial α_V cDNA was used to isolate four cDNA clones, two from the λgt10 *Xenopus* neurula stage cDNA library and two from a λZAP *Xenopus* tailbud stage cDNA library (Stratagene, Fig. 1). A 6097bp α_V cDNA sequence which contains a 3101bp open reading frame was constructed from overlapping clones (Fig. 1). The 3´ end lacks a polyA tail, which suggests that a portion of the 3´untranslated region is missing from the cloned cDNA. The sequence defines a 1033 amino acid protein with all the features typical of an integrin α subunit. A hydrophobic 19 amino acid signal peptide is followed by a large extracellular domain with multiple potential N-linked glycosylation sites (N-X-S/T), four potential cation binding sites (D-X-D/N-X-D-G-X-X-D), cysteine residues at highly conserved positions and a proteolytic cleavage site (K/R-R-E/D) located between residues 886 and 887. At the C-terminal end a single transmembrane domain of 27 amino acids is followed by a 32 amino acid cytoplasmic segment. The cytoplasmic domain contains the consensus sequence G-F-F-K/R-R, which is common to all known integrin α subunits (Fig. 2).

The amino acid sequence of the mature *Xenopus* α_V subunit was compared to the *Pleurodeles*, chicken, mouse and human α_V subunit using the BESTFIT module of the Genetics Computer Group program (Devereux *et al.*, 1984). As shown in Table 1 the mature *Xenopus* integrin α_V subunit shares considerable similarities with α_V sequences from other species. The positions of 10 of the 13 N-linked glycosylation sites (Fig. 2, asterisks) and 18 of the 19 cysteine residues (Fig. 2, bold letters, underlined) are conserved when compared to the human and chicken α_V subunit, as is the position of the putative protease cleavage site (Fig. 2, arrow).

Temporal expression of the Xenopus integrin α_V mRNA

Northern blotting with RNA from embryos of different developmental stages revealed a single transcript with a size of approximately 7.5 kb (Fig. 3A, lanes 1 and 2). α_V integrin mRNA is maternally provided and is present throughout development (Fig. 3B, lanes 1-9). The intensity of the α_V signal corresponds to that of β_1 integrin which served as loading control. Two forms of β_1 integrin are known to be present at constant levels throughout early development. The β_1 specific probe detects the two forms of the *Xenopus* β_1 integrin, β_1 and β_1* described by Gawantka *et al.*, (1992) for *Xenopus*.

Integrin α_V specific antisera

To analyze the α_V protein expression a polyclonal antiserum named $\alpha_V$fus was raised against a bacterial fusion protein (QE$\alpha_V$fus). A further antiserum named α_VQ19R was raised against a synthetic peptide (Q19R). The fusion protein contains part of the extracellular region of the α_V protein (aa 175-478) including the four metal binding domains (Fig. 2, black line). The synthetic peptide corresponds to the C-terminal 20 amino acids of the extracellular domain of the integrin α_V subunit (Fig. 2, short black bar). The antisera were affinity purified on columns containing the appropriate immobilized antigen. Western blotting and immunoprecipitation assays were performed to determine the specificity of the α_V antibodies.

Figure 4A demonstrates that the $\alpha_V$fus antiserum only recognizes the bacterially expressed α_V fusion protein (lane 1). If the α_V

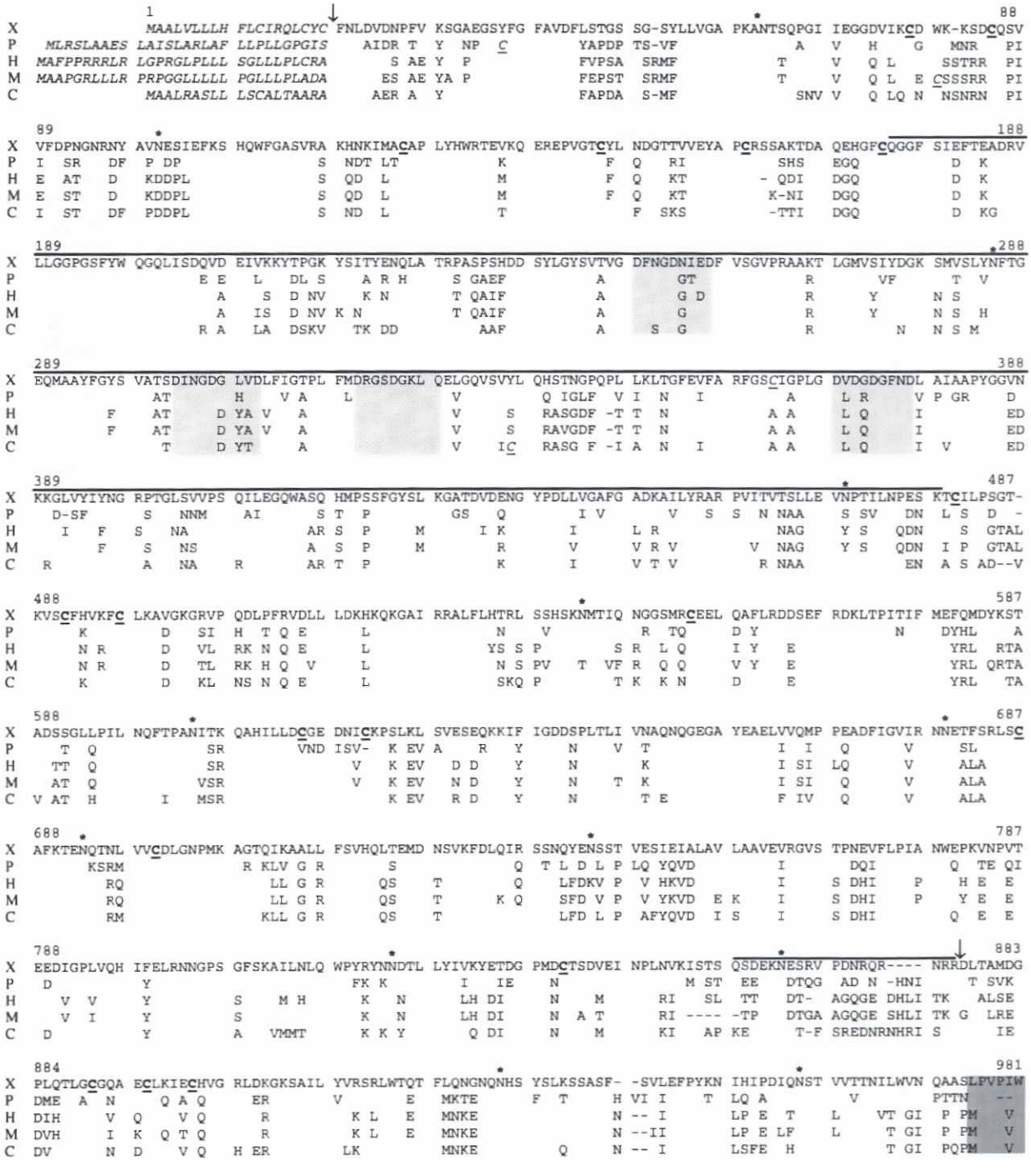

Fig. 2. Amino acid sequence alignment of the *Xenopus* (X), *Pleurodeles* (P; Alfandari *et al.*, 1995), human (H; Suzuki *et al.*, 1987), mouse (M; Wada *et al.*, 1996) and chick (C; Bossy and Reichardt, 1990) homologs of the integrin α_V subunit. *Only differing amino acids are shown and gaps are marked by dashes. The signal peptides which are less conserved between the species are in italics. The cleavage site for the generation of the mature protein is indicated by an arrow. The four conserved metal-binding domains are indicated by light grey boxes. Conserved cysteine residues appear in bold type and are underlined, a single non-paired cysteine within the Xenopus sequence is in italics and underlined, as are single cysteines within the other sequences. N-linked glycosylation sites are marked with an asterisk above the Xenopus sequence. The transmembrane domain is indicated by a dark grey box. The part of the extracellular region which was expressed as a fusion protein for immunization is indicated as a black line above the Xenopus sequence, as is the amino acid sequence of the immunogenic peptide. Amino acid numbers correspond to the Xenopus α_V amino acid sequence. The Xenopus α_V sequence has the GenBank accession no. U92006.*

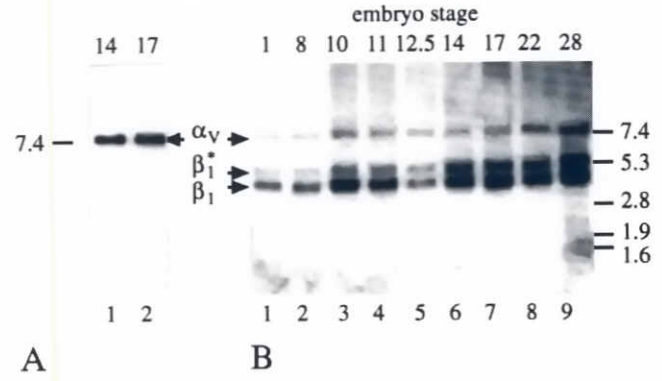

Fig. 3. α_V mRNA analyzed by Northern blots. (A) *5 μg of total RNA extracted from embryos of the developmental stages indicated at the top of each lane was hybridized to a radioactively labelled cDNA probe representing the coding region of the α_V integrin. A single transcript is found with a length of 7.5 kb.* **(B)** *The α_V hybridization was performed together with a radioactively labelled cDNA probe representing the coding region of β_1 integrin. The β_1 specific probe detects the isoforms of the Xenopus β_1 integrin, β_1 and β_1*. Stage 1, fertilized egg; stage 8, midblastula; stage 10-12.5, gastrula; stage 14-22, neurula; stage 28, tailbud. The α_V, β_1 and β_1* transcripts are marked with an arrow. The positions of the mRNA marker (1.6, 1.9, 2.8, 5.3 and 7.4 kb) are indicated at the right.*

fusion part is omitted the $\alpha_V$fus antiserum does not give a signal on the blot (lane 2). We tested whether the $\alpha_V$fus antiserum crossreacts with the α_5 subunit, because the region we chose for the preparation of the a_v fusion protein shares 56% identity with the corresponding region of the *Xenopus* integrin α_5 subunit. For this purpose, the α_V cDNA fragment in the pQE expression vector was replaced by the corresponding region of the α_5 cDNA and an α_5 fusion protein was obtained. This fusion protein was not recognized by the $\alpha_V$fus antiserum on a Western blot (lane 3).

With the peptide antiserum α_VQ19R immunoprecipitations were performed using stage 30 embryo extracts. The precipitates were separated by SDS-PAGE under reducing conditions, transferred onto nitrocellulose and probed with the $\alpha_V$fus antiserum. The $\alpha_V$fus antiserum detects a single protein with a M_r of $125x10^3$ (Fig. 4B, lane 4). Signals in the lower part of the α_VQ19R precipitate indicate IgGs which leaked from the precipitation beads. Further evidence for the specificity of the α_V antisera derives from Western blot analysis after performing SDS-PAGE under reducing and non-reducing conditions. The α_V subunit belongs to the group of integrin α subunits which are posttranslationally cleaved into a heavy and a light chain which remain associated via a disulfide bond. Under reducing conditions this disulfide bond is cleaved and the heavy and light chains become separated by SDS-PAGE. Both α_V specific antisera are directed against regions of the large extracellular domain. If embryo extracts are separated under reducing or non-reducing conditions, the α_VQ19R antiserum recognizes a protein with a M_r of $125x10^3$ for the reduced form and with a M_r of $150x10^3$ for the non-reduced form of the *Xenopus* integrin α_V subunit (Fig. 4C, lanes 7 and 8). No signal was obtained when the α_VQ19R antiserum was preincubated with the immunogenic peptide (data not shown). From these experiments we conclude that both antisera specifically recognize the *Xenopus* integrin α_V subunit.

To determine whether the α_V subunit is associated with the β_1 subunit extracts of gastrula stage embryos were immunoprecipitated with the β_1 specific mAb 8C8 (Gawantka *et al.*, 1992). Western blotting revealed that no α_V protein was found in the β_1 precipitate (Fig. 4B, lane 5). To demonstrate that the β_1 antibody indeed coprecipitates β_1 associated α subunits, the Western blot was probed with an α_5 specific antibody (Joos *et al.*, 1995). This antibody detects a protein with a molecular weight of $135x10^3$ which corresponds to the reduced form of the α_5 protein (Fig. 4B, lane 6, Joos *et al.*, 1995). Therefore the α_V subunit is presumably complexed to a subunit other than β_1 in the early *Xenopus* embryo.

Integrin α_V protein during early Xenopus embryogenesis

Western blot analysis with extracts of embryos at different stages was performed using the α_VQ19R antiserum. The α_V protein is present from the fertilized egg onwards and increases in amount after neurulation, an observation which correlates with the mRNA expression (Fig. 5A, lanes 1-9). In comparison, a significant accumulation of β_1 integrin (Fig. 5B, lanes 1-9), which was used as a loading control, already commences at the onset of gastrulation (Gawantka *et al.*, 1992).

To localize α_V integrin, embryos were immunostained and sections were inspected under the fluorescence microscope. A faint signal for α_V protein could be detected on all cells of a gastrula stage 10 embryo (Fig. 6A, arrowhead) using the very sensitive tyramide method (Vanheusden *et al.*, 1996). The former egg membrane is devoid of α_V integrin (Fig. 6A, arrow). On a transverse section of an early tailbud (stage 24) embryo a weak ubiquitous staining could be found on all cells of all three germ layers (Fig. 6B). At later stages α_V integrin accumulates in specific regions. At stage 35 staining is most conspicuous in the inner layer of the ectoderm whereas it is no longer detectable in the outer layer of the ectoderm (Fig. 7A-C, arrow). All cells of the paraxial mesoderm give a faint signal (Fig. 7A, pm). The intensity of the signal within the inner layer of the ectoderm increases

TABLE 1
PER CENT IDENTITY (SIMILARITY) BETWEEN THE XENOPUS, *PLEURODELES*, CHICK, MOUSE AND HUMAN HOMOLOGUES OF THE INTEGRIN α_V SUBUNIT

	Pleurodeles	Chick	Mouse	Human
Xenopus	73.2% (84.2%)	71.9% (83.7%)	71.3% (82.9%)	72.7% (84.2%)
Human	74.6% (84.2%)	83.5% (90.3%)	93.2% (95.9%)	
Mouse	74.4% (84.4%)	82.1% (89.1%)		
Chick	75.6% (85.5%)			

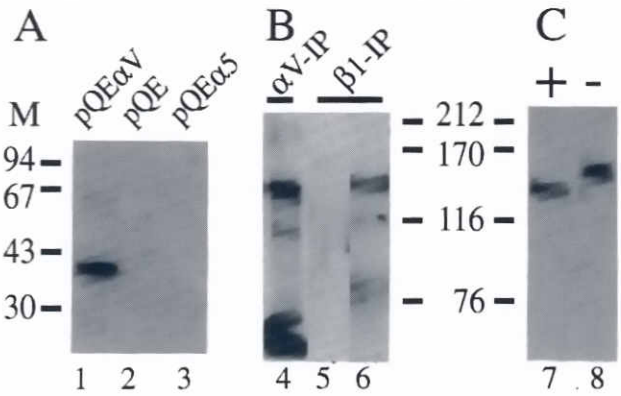

Fig. 4. Characterization of α_V specific antibodies. (A) *Immunoblot analysis of bacterial lysates with the polyclonal antibody $\alpha_V$fus. This antiserum recognizes the pQEα_V fusion protein with a M_r of 38x10^3 (lane 1) which was used as the immunogen. It does not react with an extract of bacteria containing the pQE plasmid alone (lane 2) or with the pQEa5 fusion protein in which the α_V part is replaced by the corresponding region of the α_5 integrin (lane 3).* **(B)** *Immunoprecipitations were performed, either with the α_VQ19R peptide antiserum (lane 4) or with the β_1 specific mAb 8C8 (lanes 5 and 6). The precipitates were analyzed by immunoblotting with the polyclonal antibody $\alpha_V$fus (lanes 4 and 5) or with the α_5 specific antiserum Xa5D (lane 6). α_V protein could only be detected by the $\alpha_V$fus antiserum in the α_VQ19R precipitate (lane 4), whereas in the β_1 precipitate no α_V signal was observed (lane 5). In the β_1 precipitate the α5 protein could be detected (lane 6). Signals in the lower part of the α_VQ19R precipitate (lane 4) indicate IgGs which leaked from the precipitation beads.* **(C)** *Post-translational cleavage of the Xenopus integrin α_V subunit. SDS-PAGE of embryo extracts were performed either under reducing conditions (lane 7) or under non-reducing conditions (lane 8). In the immunoblot the α_VQ19R antiserum recognizes the intact α_V protein in the non-reduced samples with a relative Mr of 150x10^3 (lane 8) and the large extracellular part with a relative M_r of 125x10^3 (lane 7).*

during further development. On transverse sections through the head and trunk of a stage 50 embryo integrin is prominent in the inner layer of the ectoderm, on endothelial cells enclosing the pharynx and the gut, and in the aorta dorsalis (Fig. 7D and E). In the eye α_V integrin was found in the retinal pigment epithelium and the retina. No staining was observed when the antiserum was preabsorbed with the antigen (Fig. 6C and data not shown).

Discussion

In this report we describe the molecular cloning of the *Xenopus* integrin α_V cDNA and present data on the presence of the α_V mRNA and protein during development. The amino acid sequence deduced from the cloned cDNAs displays a high degree of homology to the known α_V sequences from human, chicken, mouse and urodele *Pleurodeles* (Suzuki *et al.*, 1987, Bossy and Reichardt, 1990; Alfandari *et al.*, 1995; Wada *et al.*, 1996). The sequence conservation is even higher in regions involved in α_V function. The four metal binding regions, which are essential for ligand receptor interaction, are nearly 80% identical and the cytoplasmic domain, which is thought to be involved in receptor activity regulation (O'Toole *et al.*, 1994), is 83% identical to the α_V subunit of the other species. An interesting observation is the high homology score of 72% over the entire α_V amino acid sequence compared to the values that are obtained when other *Xenopus* integrin α subunits are matched to their mammalian homologs. The *Xenopus* α_3 (Meng *et al.*, 1997), α_4 (Whittaker and DeSimone, personal communication), α_5 (Joos *et al.*, 1995) and α_6 (Lallier *et al.*, 1996) subunit share only 55% to 66% identity to their mammalian homologs. This indicates that the sequence of α_V integrin is more constrained during evolution than that of other integrin α subunits. Presumably the need to interact alternatively with five or more different β subunits imposes on the α_V integrin a higher stringency for sequence conservation than on those α integrins which interact only with one or two β subunits.

We obtained no evidence for the existence of α_V integrin pseudoalleles. All cDNA fragments that we isolated from two different libraries proved to be identical within the overlapping sequences and Northern blots revealed only one size of the α_V mRNA of 7.5 kb. Thus, if a pseudoallele exists, it may either be not transcribed, or the mRNA of the two alleles must be of identical size. α_V mRNA and α_V protein are maternally provided and are present throughout development. It is currently not known which β subunit is associated with the α_V subunit during early *Xenopus* development. Although the β_1 subunit is present in the *Xenopus* oocyte and the embryo in relatively high amounts (Gawantka *et al.*, 1992; Müller *et al.*, 1993) and this subunit can dimerize with α_V (Bodary and McLean, 1990; Vogel *et al.*, 1990), the α subunit does not coprecipitate with β_1 out of extracts derived from gastrula stage embryos (Fig. 5B, Ramos *et al.*, 1996), indicating that α_V is not complexed to the β_1 subunit at this time of development. A

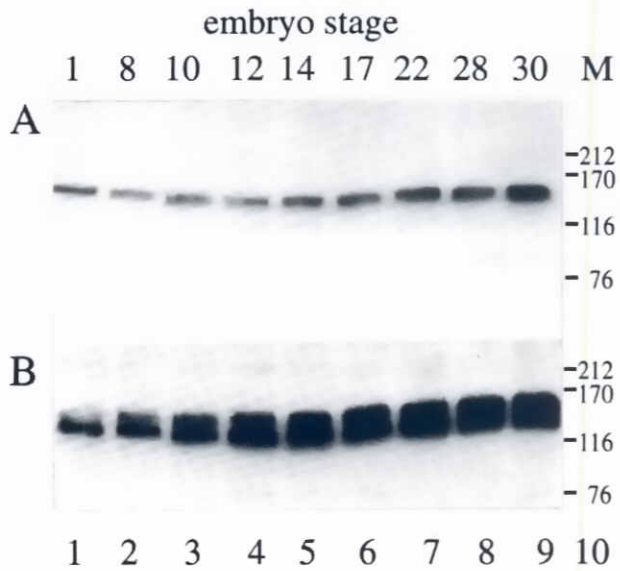

Fig. 5. Temporal expression of the Xenopus integrin α_V protein during development. *Extracts from embryos of different stages of development (five embryos per lane) were analyzed by immunoblot using the peptide specific a_V antiserum α_VQ19R* **(A)** *or a β_1 specific antiserum (Gawantka et al., 1992). The α_V protein is present throughout development with an increased expression after neurulation. A β_1 specific antiserum recognizing the β_1 precursor and the mature protein was used as a loading control (Gawantka et al., 1992). Developmental stages and the position (bars) and size (M_rx10^{-3}) of relative molecular weight markers (M) are indicated. Stage 1, fertilized egg; stage 8, midblastula; stage 10+12, gastrula; stage 14-22, neurula; stage 28+30, tailbud.*

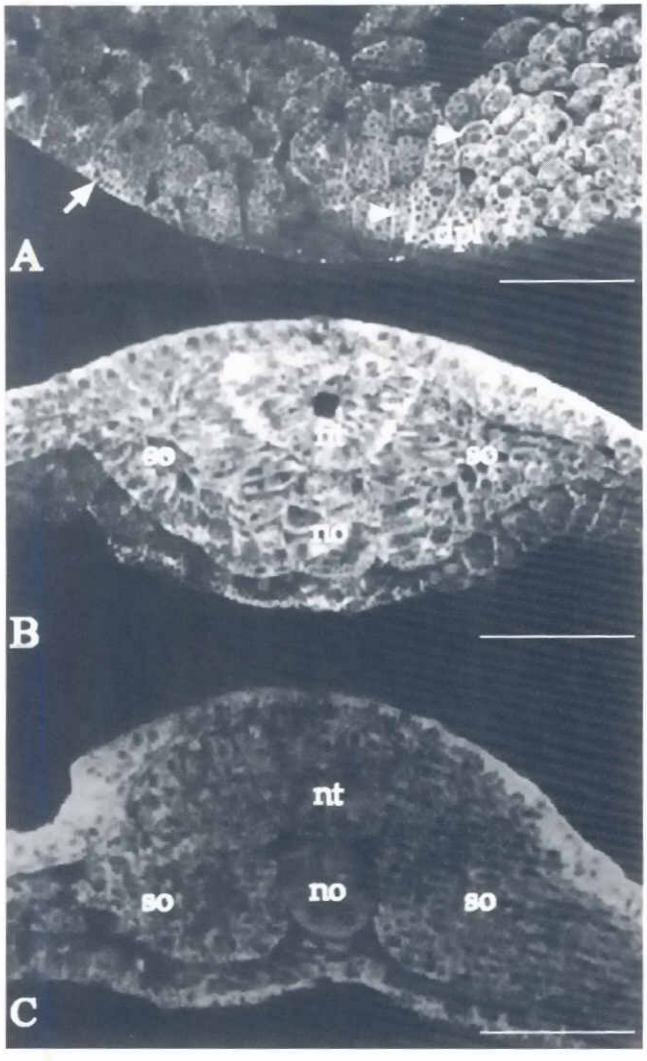

Fig. 6. Localization of the *Xenopus* integrin α_V subunit in gastrula and early tailbud stage embryos. *Vibratom sections of gastrula stage (stage 10.5) and early tailbud stage (stage 24) embryos were stained with α_V specific antibodies using the tyramide signal amplification method for visualization in the fluorescent microscope. Vibratom sections were then embedded in glycolmethacrylate and sectioned.* **(A)** *Dorsal blastopore lip region of a stage 10.5 embryo, arrow heads indicate the faint signal on the cell borders, the arrow points to the former egg membrane, which is devoid of α_V protein.* **(B)** *Transverse section of a stage 24 embryo.* **(C)** *Transverse section through a stage 24 embryo stained with the α_V specific antiserum preabsorbed with the immunogenic fusion protein. dbl, dorsal blastopore lip region; nt, neural tube; no, notochord; so, somites. Bars, 100 μm.*

good candidate for the formation of a heterodimer with α_V at the gastrula stage is the integrin β_3 subunit as suggested by Ramos *et al.*, (1996). However, this has to be confirmed with a *Xenopus* β_3 specific antibody, which is not yet available.

In gastrula and neurula stage embryos α_V protein was found on all cell membranes (Fig. 6) but the amount is much lower than that observed for the $\alpha_5\beta_1$ fibronectin receptor (Gawantka *et al.*, 1992; Joos *et al.*, 1995). Similar to the α_5 distribution, α_V is absent from the former egg membrane and disappears from the outer layer of the ectoderm at later stages. Strong expression of α_V integrins within the inner layer of the ectoderm was observed at late tadpole stages, whereas α_3 and α_5 integrins are already prominent there at late neurula stages (Gawantka *et al.*, 1994; Joos *et al.*, 1995). All three integrin subunits could function as fibronectin receptors in the emerging basal lamina (Krotoski and Bronner-Fraser, 1990). Another potential α_V ligand is thrombospondin-4, whose mRNA is expressed at this stage of development (Lawler *et al.*, 1993). Aybar *et al.* (1996) reported the presence of a vitronectin-like protein in oocytes of the amphibian *Bufo arenarum*, which could be the appropriate ligand for integrin α_V during early development. But so far, no vitronectin has been found in the *Xenopus* embryo. However in a cell culture assay it was shown that *Xenopus* myotomal myocytes can integrate vitronectin from the surrounding medium in their basal lamina (Anderson *et al.*, 1996). The observation that the α_V antibody stains the retina and the endothelium of the aorta may be seen in relation to α_V function in neurite outgrowth and angiogenesis (Neugebauer *et al.*, 1991; Brooks *et al.*, 1994). Despite reports that α_V integrins participate in neural crest migration (Delannet *et al.*, 1994) and nephric development (Wada *et al.*, 1996) no prominent α_V expression was found in these regions in the *Xenopus* embryo. In *Pleurodeles* integrin α_V protein is very prominent during early development with its amount slightly decreasing from egg to early tadpole stage and starting to increase again at larva stage (Alfandari *et al.*, 1995). In the *Xenopus* embryo only a small amount of α_V protein is found in the fertilized egg, which increases steadily during further development. While in *Xenopus* during gastrulation α_V is evenly distributed in all three germlayers, in *Pleurodeles* it is found to be more prominent on migrating mesodermal cells (Alfandari *et al.*, 1995). The authors speculate on the involvement of α_V integrin in the migration of these cells on the fibronectin containing matrix of the blastocoel roof (Alfandari *et al.*, 1995). Whether this difference in the distribution of α_V integrin between *Xenopus* and *Pleurodeles* correlates with a difference in function could be clearly addressed if function blocking antibodies for both species were available, which is not yet the case.

Recently it was reported that cells expressing a cytoplasmic truncated version of α_V integrin were deficient in adhesion to fibronectin and vitronectin (Filardo and Cheresh, 1994). Overexpression of a cytoplasmically truncated version of the α_V subunit in the *Xenopus* embryo has no influence on normal development (Joos and Hausen, unpublished results). Whether the overexpressed truncated α_V subunit does not at all interfere with α_V function in the *Xenopus* embryo or the endogenous α_V protein is sufficient for normal development could not be decided. Another possibility is that other integrins, like for example $\alpha_5\beta_1$ (Gawantka *et al.*, 1992; Joos *et al.*, 1995), which is expressed on all cells of the gastrula stage embryo, can substitute α_V function. However, targeted mutations of different integrin fibronectin receptors with the methods of homologous recombination in embryonic stem cells produce different phenotypes, which suggests that the different integrins do have independent functions and cannot completely substitute each other (Hynes, 1996). But the receptor elimination experiments also reveal that there is an overlap of functions and mutual compensation, for example α_V and α_5 integrins can both assemble a fibronectin matrix (Akiyama *et al.*, 1989; Yang *et al.*, 1996) and none of the fibronectin receptor mutations is as severe as the phenotype of the mutation of the ligand fibronectin (Hynes, 1996). Whether in the *Xenopus* embryo

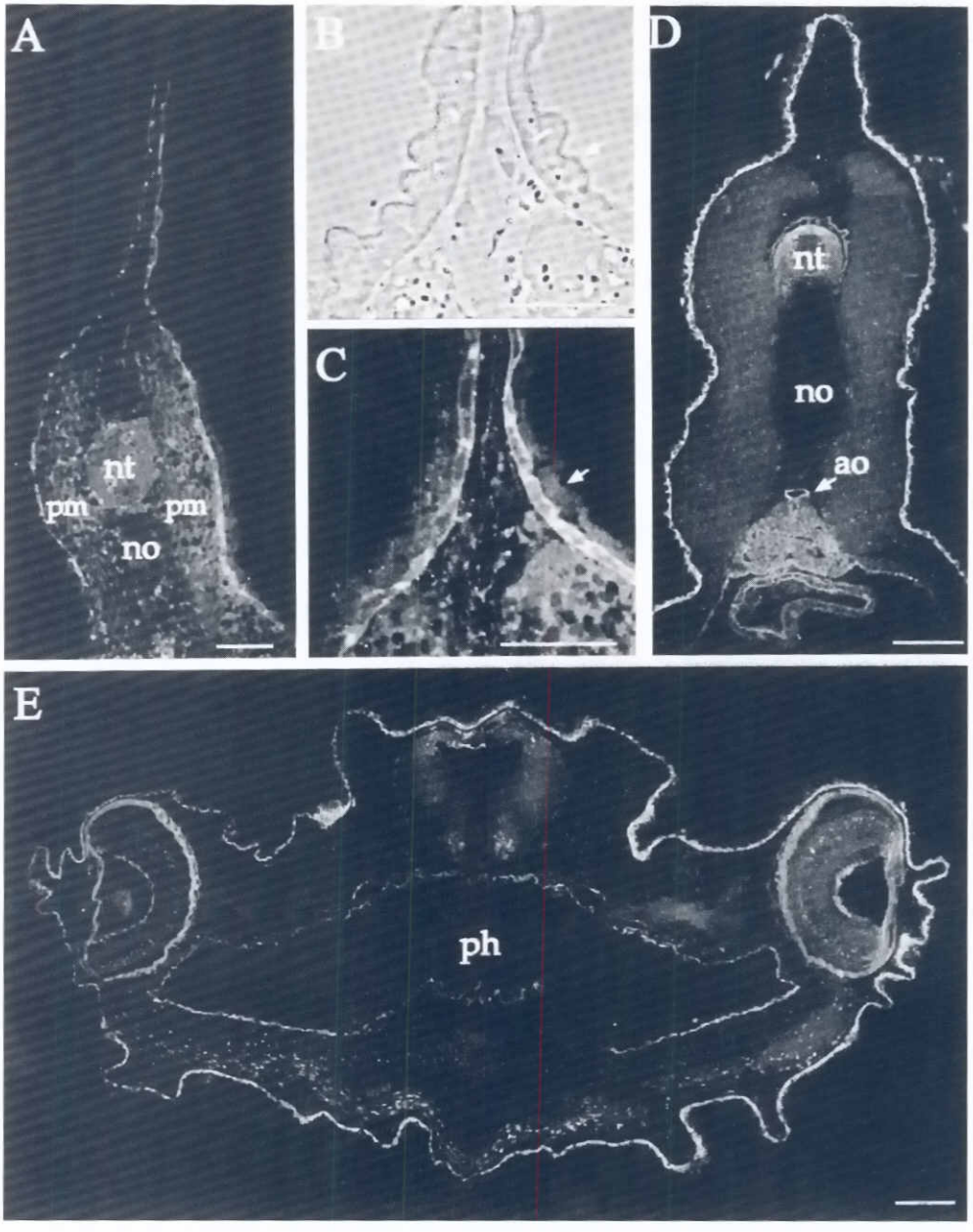

Fig. 7. Distribution of the *Xenopus* integrin α_V subunit in tadpole stage embryos. *Embryos were whole-mount stained with anti-α_V antiserum, embedded in glycolmethacrylate and sectioned.* **(A)** *Transverse section of a stage 35 embryo.* **(B)** *phase contrast and* **(C)** *Immunofluorescence of the dorsal region of a stage 35 embryo at higher magnification. The arrow points at the outer layer of the ectoderm* **(D)** *Transverse section through the trunk of a stage 50 tailbud.* **(E)** *Transverse section through the head of a stage 50 tailbud embryo. pm, paraxial mesoderm; nt, neural tube; no, notochord; ao, aorta dorsalis; ph, pharynx; Bars, 100 μm.*

these fibronectin receptors exhibit overlapping functions will be the focus of further experiments.

Materials and Methods

Embryos

Adult *Xenopus laevis* were purchased from the African *Xenopus* Facility C.C. (South Africa). Adult albino *Xenopus laevis* were obtained from *Xenopus* 1 (Ann Arbor, MI). Embryos were obtained by *in vitro* fertilization and staged according to Nieuwkoop and Faber (1967).

PCR amplification of α_V cDNA fragments

A 138bp α_V specific cDNA fragment was amplified via PCR using the same primers and conditions as described in Joos *et al.*, 1995. An α_V gene specific primer was synthesized (reverse: 5′-TAGTGATAACAGCAGCAAGCC-3′). 100 ng of this α_V gene specific primer was used together with 100 ng λ arm specific primer (forward: 5′-AGCAAGTTCAG CCTGGTTAAGTC-3′), with 1 μg template DNA and 1 unit of Amplitaq (Cetus-Perkin-Elmer), 10 mM Tris-HCl pH 8.2, 50 mM KCl, 2.5 mM MgCl, 0.1% (w/v) gelatin and 2.5 mM dNTPs under the following conditions: [94°C, 1 min], [62°C, 1 min], [72°C, 3.5 min], 30 cycles followed by a final extension step of ten minutes at 72°C. The amplified cDNA was subcloned into pBluescript SK+ (Stratagene) and sequenced with an A.L.F. Sequencer (Pharmacia).

Isolation and characterization of cDNA clones

A 372bp α_V cDNA fragment was Digoxigenin-dUTP labeled using a DIG-PCR labeling kit (Boehringer Mannheim Biochemical). With this probe $3x10^5$ pfu of a stage 17 λgt10 cDNA library (Kintner and Melton, 1987) and $3x10^5$ pfu of a stage 30 Uni-ZAPTM XR Library (Stratagene) were screened under high stringency (DIG EASY HybMix, Boehringer Mannheim Biochemical) at 39°C overnight, followed by washing 2x5 min at room temperature in 2x SSC, 0.1% SDS and 3x15 min at 65°C in 0.2x SSC, 0.1% SDS. From single phage clones either *in vivo* excision of the pBSK phagemid was performed or inserts were isolated and cloned into pBluescript SK+ (Stratagene). Sequencing was performed with an A.L.F. Sequencer (Pharmacia) either from restriction site generated subclones using primers supplied by the manufacturer or with gene specific primers synthesized from Pharmacia. Sequence analysis was performed with the GCG software package (Devereux *et al.*, 1984).

mRNA analysis

Total RNA from embryos was isolated as described by Ellinger-Ziegelbauer and Dreyer (1991). RNA was separated on denaturing formaldehyde agarose gels and transferred to "GeneScreen" nylon filters (DuPont). Two embryo equivalents were loaded per lane. cDNA probes that represent nt 38 to 3139 of the *Xenopus* α_V subunit and nt 78-3500 of the *Xenopus* β_1* cDNA (Gawantka *et al.*, 1992) were ^{32}P-labelled by random priming and were hybridized to the filter overnight at 65°C. Filters

were washed in 2xSSPE/0.1% SDS (1xSSPE=150 mM NaCl, 10 mM $NaH_2PO_4(2H_2O)$, 10 mM EDTA, pH 7.4) for 2x15 min and in 0.1xSSPE/0.1% SDS for 3x30 min. Autoradiographs were obtained by exposure onto Cronex Northern (DuPont) X-ray film at -70°C.

Polyclonal anti α_v antibodies

A 19 amino acid oligopeptide (NH_2-QSDEKNESRVPDNRQRNRR-COOH) corresponding to the predicted C-terminus of the large extracellular fragment and a fusion protein which contains the extracellular region aa174-aa479 of the α_v integrin were used to immunize rabbits. For the fusion protein a part of the α_v cDNA was amplified with PCR. The PCR was performed using an α_v forward primer with a BamHI recognition site (558se: 5´-CGGGATCCAAGGGGGGTTTAGTATTG-3´) and an α_v reverse primer with a SalI recognition site (1427as 5´-GGCCCGTCGAC TGTTTTACTTTCTGGGTTTAAG-3´), pfu polymerase (Stratagene) and 100 pg of the plasmid pBα_v1-3601 DNA as template. The amplified PCR product was BamHI/SalI digested, gel purified and cloned into pQE9 (Qiagen, Germany). Production and isolation of the fusion protein was performed according to the manufacturer's protocol. Purification was performed under denaturing conditions on a Ni-charged nitriloacetic acid column (Qiagen, Germany). Either 0.25 mg peptide coupled to keyhole limpet hemocyanin (KHL) or 0.3 mg fusion protein emulsified with complete Freud´s adjuvants were used for the immunization of rabbits. Two additional boosts were performed at four weeks´ intervals with the same amount of antigen using incomplete Freund´s adjuvant. Sera were collected weekly after the final booster until the titer decreased. Affinity purified antibodies were obtained by passing 5 ml of the serum, diluted in 10 ml PBS, through a MiniLeak™ (Ken-Em-Tec) column coupled with the immunogenic antigen. The antibodies were eluted with 0.1 M glycine pH 2.5 and neutralized with 1/50 Vol. of 2 M Tris-HCl pH 7.6. The purified peptide antiserum was named α_vQ19R and the fusion protein antiserum $\alpha_v$fus.

Westernblotting and immunohistochemistry

Westernblotting and immunohistochemistry was performed as described in Joos *et al.*, (1995).

For immunoprecipitation experiments 10 µg of the respective antibody was bound to 20 µl Protein A- Sepharose for rabbit antibodies or to Protein G-Sepharose for mice antibodies (Pharmacia, Germany). Extracts for immunoprecipitations were centrifuged and filtered through a 0.22 µm filter and incubated for one hour with the Protein A-sepharose-coupled antibodies. The precipitates were washed extensively with lysisbuffer and bound proteins were eluted with SDS-sample buffer and subjected to SDS-PAGE and western blotting.

Tyramide Signal Amplification (NEN™ Life Science Products, Boston) was performed according to the Manufacturer's protocol. Briefly, DMSO fixed embryos were incubated with α_v specific antibodies (1 µg/ml) diluted in PBS 20% NDS/0.1%Sap (20% normal donkey serum/0.1% saponin) over night, washed 6x1 h in PBS/0.05% saponin at room temperature and fixed for 1 h at room temperature with 3.7% formaldehyde and 0.1% glutaraldehyde. From these embryos 150 µm vibratom sections were cut and incubated with biotinylated Goat-anti-rabbit antibody (Dianova) over night, washed 6x15 min in TNT buffer, blocked in TNB buffer (NEN™), incubated with Streptavidin-HRP (NEN™) for 1 h followed by 6x15 min washes. Samples were incubated 10 min with Fluorophore Tyramide (NEN™) and washed 5x10 min in TNT buffer. After dehydration with 3,2 dimethoxypropane (2x3 h) the specimens were embedded in glycolmethacrylate (Technovit 7100, Kulzer, FRG) and 5 µm sections were prepared on a Reichert Jung 1140 autocat microtome. Images were made with a digital camera DXC-950P (Sony) and processed using Adobe Photoshop 4.0.

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