Biosynthesis and In Vivo Localization of the Decapentaplegic-Vg-related Protein, DVR-6 (Bone Morphogenetic Protein-6)

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Abstract. DVR-6 (BMP-6 or Vgr-1) is a member of the TGF-β superfamily of polypeptide signaling molecules. In situ hybridization studies have previously shown that DVR-6 RNA is expressed in a variety of cell types in the mouse embryo, but no information has been available on protein localization and biosynthesis. We have produced a polyclonal antibody to the proregion of DVR-6 and used it to localize the protein in whole mount and sectioned embryonic, newborn, and adult mouse tissues. DVR-6 protein is expressed in the mouse nervous system beginning at 9.5 days postcoitum (d.p.c.) and continues through adulthood. A variety of epithelial tissues also produce DVR-6 protein, including the suprabasal layer of the skin, bronchiolar epithelium, and the cornea. Additionally, a stably transfected cell line, BMGE+H/D6c4, is used to study the biosynthesis of DVR-6 protein and evidence is presented for translational regulation of DVR-6 expression.

1. Abbreviations used in this paper: BMGE, bovine mammary gland epithelial; BMP, bone morphogenetic protein; d.p.c., days postcoitum; DPP, Drosophila decapentaplegic; DVR, decapentaplegic-Vg-related; GST, glutathione-S-transferase; TGF-β, transforming growth factor beta.
Materials and Methods

Antigen Production

A glutathione-S-transferase (GST)/DVR-6 fusion protein was made using the pGEX system described by Smith and Johnson (1988). A 256-bp XhoII/RsaI fragment (nucleotides 180 to 436) of the mouse DVR-6 cDNA designated Vgr-1 (Lyons et al., 1989a) encoding part of the proregion was inserted into the Smal site at the 3’ end of the GST-coding region in the pGEX-2T vector. This region of Vgr-1 was chosen because it encodes the part of the DVR-6 protein that has diverged most extensively from the closely related proteins DVR-5 and DVR-7 (BMP-5 and BMP-7, Celeste et al., 1990). Comparison of the predicted amino acid sequence of hDVR-6 with that of hDVR-5 and hDVR-7 in the region used for making the GST/DVR-6 fusion protein reveals only 56.8 and 33.3% identity, respectively (Celeste et al., 1990).

Preparation of antigen is described elsewhere (Wall et al., 1992).

Antibody Preparation and Specificity

Female New Zealand rabbits were injected initially subcutaneously and intramuscularly in a total of four sites with a total of 750 µg of GST/DVR-6 fusion protein emulsified in Freund’s complete adjuvant. Approximately 24% of this total represents DVR-6 protein. Subsequently, 750-µg boosts were given in Freund’s incomplete adjuvant. Antibody preparation and specificity were determined by previously described methods (Wall et al., 1992).

In Vitro Transcription of DVR-5, -6, and -7 RNA

The full-length human DVR-5, -6, and -7 cDNAs were used for in vitro transcription of RNA. Linearized DNA template (inserted behind an SP6 promoter) was incubated with the following: 5 mM m7G(5’)ppp(5’)G (Pharmacia LKB Biotechnology Inc., Piscataway, NJ); 5 mM rATP, rCTP, and rUTP (Promega Corp.) and 2 µl ail-trans [35S]methionine (1,000 Ci/mmol; ICN Biomedicals). Translation buffer, rabbit reticulocyte lysate, and 1 mM 0.01 M Tris.HCl, pH 7.4. DVR-6 protein was detected by incubation with affinity-purified primary antibody diluted 1:1,000 in TBST followed by incubation with a 1:5,000 dilution of goat anti-rabbit IgG conjugated to peroxidase (Cappell Laboratories, Malvern, PA, 0100-1221). All antibodies were preincubated with mouse liver acetone powder before use (Harlow and Lane, 1988). The luminol solution from Amersham’s ECL kit (RPN2109) was used as a substrate for peroxidase, and the resulting chemiluminescent reaction was detected on XAR-5 film (Eastman Kodak Co., Rochester, NY).

Fixation and Immunohistochemical Staining of Embryos and Cells

Fixation of embryos is described elsewhere (Wall et al., 1992). Cells were cultured on glass coverslips, fixed in methanol/DMSO (4:1) 2 min at room temperature, treated to inhibit endogenous peroxidase activity, rinsed in PBS, and used immediately for immunohistochemistry. Immunohistochemical methods used on whole and sectioned embryos are described elsewhere (Wall et al., 1992).

Results

Specificity and Species Crossreactivity of αDVR-6

The amino acid sequence of the DVR-6 portion of the GST/DVR-6 fusion protein used for immunization (see Materials and Methods) was compared to sequences in the EMBL database (Brutlag et al., 1990) to determine if there were any other known proteins which might be recognized by the antibody. No protein sequences showed more than 22% similarity. To confirm that αDVR-6 does not cross-react with its closest family members, immunoprecipitation experiments were carried out using human DVR-5, -6, and -7 (BMP-5, -6, and -7) protein synthesized in vitro. Fig. 1 shows that αDVR-6 only recognizes DVR-6 protein.

Further evidence demonstrating the specificity of the DVR-6 antibody is shown in Figs. 2 and 3. BMGE cells
Figure 1. In vitro translation of DVR-5, -6, and -7 RNA and immunoprecipitation of translation products with αDVR-6. (Lanes 1–3) Human DVR-5, -6, and -7 protein, respectively. (Lanes 4–6) Immunoprecipitation, using αDVR-6, of human DVR-5, -6, and -7, respectively. The positions of protein markers are shown in kilodaltons on the right.

Figure 2. Immunohistochemistry of BMGE cells stably transfected with hDVR-6 using (a) αDVR-6 and (b) normal rabbit serum. n, nucleus. Bars, 20 μm.

Figure 3. Immunoprecipitation of newly synthesized protein made by BMGE (lanes 1–4) and BMGE/D6c4 (lanes 5–8) cells with αDVR-6. (Lanes 1 and 5) Medium + normal rabbit serum; (lanes 2 and 6) medium + αDVR-6; (lanes 3 and 7) cytoplasmic fraction + normal rabbit serum; and (lanes 4 and 8) cytoplasmic fraction + αDVR-6. The positions of protein markers are shown in kilodaltons on the right.

Figure 4. RNase protection of mouse DVR-6 in PYS cells. (Lane 1), 256-bp XhoI/RsaI probe; (lane 2), 512-bp protected fragment; and (lane 3), probe with water as a negative control.

Expression in Cell Lines
Lyons et al. (1989a,b) showed, using Northern analysis, that DVR-6 (Vgr-1) RNA is expressed in three cell lines—murine PYS, human 49F, and human HT1080. However, of these three, immunoreactivity for αDVR-6 is only seen in the cytoplasm of the HT1080 cell line (data not shown). This discrepancy between mRNA and protein expression could have several explanations. For example, there may be alternative splicing of the DVR-6 transcript in PYS and 49F cells that removes the region encoding the epitope recognized by αDVR-6. However, results of RNase protection assays demonstrate that the RNA sequence encoding the epitope recognized by αDVR-6 is present in PYS cells (Fig. 4). Another explanation is that translation of the mRNA is inhibited, perhaps through 5′ or 3′ untranslated sequences. Preliminary experiments which support this idea show that an hDVR-6 construct, without any 5′ or 3′ untranslated sequences, is translated when transfected into PYS cells. Other explanations are that the translation product is complexed with another protein, obscuring the epitope recognized by αDVR-6, or that DVR-6 protein is quickly degraded upon synthesis. Experiments to distinguish between these possibilities are currently under way.

Biosynthesis of DVR-6 Protein
Biosynthesis of hDVR-6 protein was examined using the stably transfected cell line, BMGE+H/D6c4. The predicted relative molecular mass of the precursor protein is 57,207 and the in vitro–translated (therefore, unmodified) DVR-6 protein is ~58,000 (Fig. 1). Fig. 3 shows that newly synthe-
Western blot of 12.5-d.p.c. mouse tissue with α-DVR-6. (Lane 1) Head; (lane 2) liver; (lane 3) spinal cord; and (lane 4) limb bud. The positions of protein markers are shown in kilodaltons on the right.

Expression of DVR-6 in the Developing Mouse Nervous System

Using whole mount immunohistochemistry with mouse embryos, DVR-6 protein is first detected at 9.5 d.p.c., in the developing trigeminal mesencephalic tracts, cranial nerves and ganglia, and spinal nerves of the anterior spinal cord (Fig. 7 a). Immunoreactivity was not seen in 9.0 d.p.c. embryos. By 10.5 d.p.c., as neural development proceeds, expression is maintained in these areas and is also seen in the spinal nerves emerging from more posterior regions of the spinal cord (Fig. 7 b and c).

Localization of DVR-6 Protein In Vivo

Previous studies using in situ hybridization had revealed DVR-6 RNA in a number of embryonic tissues including the central nervous system, skin, hypertrophic cartilage, and primary oocytes. Intact and sectioned embryos were studied by immunohistochemistry to see if localization of DVR-6 protein correlates with previously published RNA expression patterns (Lyons et al., 1989a,b).

Localization of protein was studied in more detail in transverse sections of embryos at 9.5, 10.5, 12.5, and 15.5 d.p.c. As the nervous system develops, the number of DVR-6-positive cells increases (Fig. 8). Immunoreactivity at 9.5 d.p.c. in the anterior regions of the spinal cord is in cells located in lateral and ventral regions of the marginal zone. DVR-6 protein is also seen in putative neural crest cells that presumably will contribute to the formation of the cranial ganglia. By 10.5 d.p.c., the cranial and dorsal root ganglia and the presumptive motor neurons are immunoreactive and express DVR-6 protein in cell bodies as well as axons. This is particularly evident in Fig. 8 e. At 12.5 d.p.c., both the cell bodies throughout the spinal cord and the axonal tracts which form the white matter are heavily stained, again showing that DVR-6 protein is present in axons as well as cell bodies and, therefore, is probably transported and secreted axonally. However, by 15.5 d.p.c., the staining of axon tracts is reduced.

In newborn mice, expression is in most regions of the brain but is more obvious in areas which contain larger cells or more concentrated neuronal populations such as the cere-
Figure 7. Immunohistochemistry of intact 9.5- and 10.5-d.p.c. mouse embryos. (a) 9.5-d.p.c. mouse embryo with normal rabbit serum. (b) 9.5-d.p.c. mouse embryo. Arrows indicate the developing spinal nerves. tm, trigeminal mesencephalic tract. (c) 10.5-d.p.c. mouse embryo. Cranial nerves are numbered. Arrow shows the trigeminal mesencephalic tract. ot, otic vesicle; lb, limb bud. (d) Boxed area in c. Arrows show the ventral motor roots of the spinal nerves. Asterisks show the dorsal roots of the spinal nerves. Bars: (a–c) 500 μm; (d) 100 μm.
bellum, hippocampus, and brain nuclei (data not shown). Fig. 9 shows that this expression is maintained in the adult. DVR-6 protein immunoreactivity is seen in the cerebellum (Fig. 9, a and c) and in the hippocampus (Fig. 9, b and d). While protein can be definitely localized to neuronal cells, we cannot at present exclude the possibility that it is also expressed in some glial cells. However, in counterstained sections of the eye it was apparent that there were glial cells which did not express DVR-6 protein. Additionally, in the adult, but not in embryos or newborns, αDVR-6 immunoreactivity is seen in the choroid plexus (Fig. 9 b) and meninges (data not shown).

Fig. 9 also shows the developing eye (Fig. 9 e) and cochlea (Fig. 9 f) of a 15.5-d.p.c. embryo. As seen in the spinal cord, DVR-6 immunoreactive cells are limited to the most mature region of the retina and the axon tract of the optic nerve, indicating that this protein is expressed in postmitotic neurons. In the cochlea, expression of DVR-6 protein is seen in the eighth cranial nerve and the region containing hair cells in the cochlear duct wall.

Expression in Epithelia
DVR-6 protein is first detected in the epidermis at 15.5 d.p.c., specifically in the nasal epithelium, when it is beginning to thicken and keratinize (Fig. 10, a–d). By 16.5 d.p.c., expression is seen in the suprabasal cells of the skin, but is absent from the basal layer and hair follicles. This correlates with the expression of DVR-6 mRNA (Lyons et al., 1989a,b). In newborn mice, αDVR-6 immunoreactivity is
still seen in the suprabasal layer of the epidermis. Additionally, all of the corneal epithelial cells of the adult are DVR-6 immunoreactive, but not the limbus (Fig. 10, g–i). Since the epithelial cells of the cornea migrate centripetally from the limbus, this expression pattern is analogous to that of the skin in which the basal cell layer (mitotic region) does not express DVR-6 protein, while the suprabasal layer (postmitotic) does express DVR-6 protein. Fig. 10, e and f, shows that DVR-6 protein also is expressed in the bronchiolar epithelium of newborn lung but not in alveolar epithelium.

Other Tissues

Hypertrophic cartilage, uterine stroma, and oocytes, tissues that previously had been shown by in situ hybridization to express DVR-6 mRNA, were also examined by immunohistochemistry. Hypertrophic cartilage in late-stage embryos was not immunoreactive for αDVR-6. Ovaries and uteri from each day of the estrous cycle were stained but showed no immunoreactivity in the oocytes or uterine stroma. Additionally, both fertilized and unfertilized ova taken at various
times after ovulation were also immunostained, but again showed no immunoreactivity with αDVR-6.

**Discussion**

The availability of a specific antibody for DVR-6 has provided a means for in-depth studies of its synthesis, post-translational modification, secretion, and localization in at least three species (mouse and human, this paper; chicken, Kuratani, S., unpublished observations). Although in situ hybridization studies have previously shown expression patterns for DVR-6 during development, these studies only establish where mRNA is located, not the protein. Since proteins are the agents of function, and since the presence of a transcript does not necessarily indicate translation, a protein expression pattern, as well as characterization of the protein, is important for examining the potential role of DVR-6 in the developing embryo.

Immunoprecipitations done with αDVR-6 reveal two newly synthesized protein doublets of ~62,000/65,000 and ~40,500/43,500 M_0 in cells transfected with hDVR-6 and in tissues of developing mouse embryos (Fig. 3 and data not shown). Since αDVR-6 only recognizes the proregion of the protein, it is likely that the higher doublet is the full-length DVR-6 protein and the lower is the cleaved proregion. This is consistent with the model that DVR-6 protein, as a member of the TGF-β superfamily, is synthesized as a prepro secreted protein (Brunner et al., 1988; Gentry et al., 1988; Sha et al., 1989).

DVR-6 protein, which has five potential N-glycosylation sites (two in the proregion and three in the mature region), exists in two glycosylated forms and at least its proregion is secreted from cells. The significance of two glycosylated forms of this protein is presently unknown, but it appears that glycosylation may affect secretion of DVR-6 protein since tunicamycin treatment of BMGE+H/D6c4 cells shows that secretion of the cleaved proregion of DVR-6 is inhibited when it is unglycosylated (Fig. 6). Absence of glycosylation...
may or may not inhibit the secretion of the presumed mature region of DVR-6 protein. An antibody to the mature region is currently being produced so that this can be determined.

In situ hybridization studies have shown that DVR-6 mRNA is expressed in epithelia and neural tissues, hypertrophic cartilage, and primary oocytes (Lyons et al., 1989a,b). Although αDVR-6 recognizes protein in neural and epithelial tissues, it does not detect DVR-6 protein in hypertrophic cartilage or oocytes. This discrepancy between mRNA and protein expression is also found in cell lines that contain DVR-6 transcripts. While HT1080 cells express both DVR-6 mRNA and protein, PYS and 49F cells, which contain DVR-6 transcripts, do not contain detectable protein. One explanation for this is that the 256-bp sequence encoding the epitope recognized by αDVR-6 is spliced out of the DVR-6 transcripts in these cell lines and tissues. However, RNase protection experiments using this 256-bp sequence as a probe in PYS cells show that the sequence is present (Fig. 4). Therefore, a more plausible explanation for this discrepancy is that DVR-6 mRNA is not translated in these tissues and cell lines. However, we cannot rule out the possibility that other mechanisms may be involved. DVR-6 protein could be degraded as it is translated or it could complex with another protein masking the epitope recognized by αDVR-6. To explore the possibility of translational control, PYS cells, a cell line in which DVR-6 transcript but not protein is detected, have been stably transfected with hDVR-6. The hDVR-6 construct excludes 5' and 3' untranslated sequences since these may be involved in translational control (Hershey, 1991). Preliminary results demonstrate that PYS cells are capable of synthesizing DVR-6 protein (data not shown). This suggests that translational control via 5' or 3' untranslated sequences has been relieved. In the embryo, it remains to be seen whether translational control plays a role in the lack of DVR-6 protein expression in hypertrophic cartilage and oocytes.

Immunohistochemical staining of epithelial and neuronal tissues shows that DVR-6 protein is expressed in vivo at different stages of development. In the epidermis, protein is first detected at 15.5 d.p.c., when the epidermal thickening is beginning to occur, in the suprabasal layers of the nasal region. By 16.5 d.p.c., expression is seen in the suprabasal layer of the skin and this pattern is maintained in newborns. Also in newborns, bronchiolar epithelium is immunoreactive for αDVR-6. Additionally, in the adult, the corneal epithelium, but not the limbus or conjunctival epithelium, expresses DVR-6. This is noteworthy because, based on keratin expression, the limbal and conjunctival epithelia more closely resemble the basal layer of the skin while the corneal epithelium resembles the suprabasal layer (Schermer et al., 1986). The role of DVR-6 in these epithelial tissues is unknown at this time. However, since the suprabasal and corneal epithelia are differentiated, postmitotic cells, secreted DVR-6 protein may act in an autocrine manner to promote withdrawal from the cell cycle and/or differentiation. Alternatively, DVR-6 may be acting in a paracrine fashion to regulate the proliferation and differentiation of basal cells.

In the nervous system, DVR-6 immunoreactive cells are first detected at 9.5 d.p.c. in the trigeminal mesencephalic tract, cranial ganglia, and spinal cord. Also at this stage, both the sensory and motor components of the spinal nerves begin expressing protein, first anteriorly, and then in more posterior sites as development proceeds. Protein is seen in both the cytoplasm and axons of neuronal cells at this time. This expression is particularly evident in the motor neurons of the 10.5-d.p.c. embryo (Figs. 7 and 8). Cross sections of spinal cord from stages 9.5-15.5 d.p.c. and sagittal sections of brain from stages 12.5-adult (data not shown) reveal that immunoreactive cells first appear in the outer layers, and then subsequently more interior layers of these tissues. This temporal and spatial expression pattern suggests that DVR-6 is expressed in newly born, postmitotic neurons. This hypothesis will be tested by correlating DVR-6 expression with bromodeoxyuridine labeling.

Flanders et al. (1991) have shown that TGF-β-2 and TGF-β-3 proteins are expressed in most regions of the embryonic nervous system, but not as extensively as DVR-6 protein. For example, while DVR-6 is present in all cranial and dorsal root ganglia, TGF-β-1, 2, and 3 are absent from some of these ganglia. Another difference in expression of these proteins is that TGF-β is present in some glia in vivo and in vitro (Flanders et al., 1991; Saad et al., 1991; Constam et al., 1992; Lindholm et al., 1992) but, so far, no expression of DVR-6 has been detected in a number of glial cell lines studied (data not shown). Additionally, preliminary studies in which sections of the 15.5-d.p.c. optic nerve have been immunostained and counterstained, do not show staining of DVR-6 in cells presumed to be glia associated with the nerve tract (data not shown). If DVR-6 proves to be neuron specific, it can be used as an early marker distinguishing between neuronal and glial cells.

The presence of DVR-6 protein in the cell bodies and axons of postmitotic cells of the nervous system suggests a variety of potential functions, either paracrine or autocrine in nature. Since there is presumably a secreted form of the protein, one potential function is the stimulation or inhibition of cell division or the promotion of differentiation, of associated glial cells, neighboring neurons, innervating neurons, or target cells, such as muscle. Further studies to determine whether DVR-6 is secreted via dendrites or axons (or both), and, like other members of the TGF-β superfamily, it is sequestered by the extracellular matrix, will help in determining the role of this protein in neural development. It will be necessary to produce purified protein and an antibody directed against the mature protein (experiments are currently under way) before these questions can be fully addressed. Also, identification and study of a receptor for DVR-6 will aid in elucidating the function(s) of this protein.

Immunoprecipitation and Western blot analysis was done to rule out the possibility that αDVR-6 cross-reacts with a neurofilament protein. Immunoprecipitation experiments of 12.5-d.p.c. brain and spinal cord showed that the uncleaved form of DVR-6 protein was being synthesized, but at a low rate (data not shown), and the results of Western analysis show that protein of the expected relative molecular mass for the cleaved proregion of DVR-6 is present (Fig. 4). This demonstrates that the appropriate size protein is present in tissue extracts and that for steady-state protein levels, the predominant form of DVR-6 protein recognized by αDVR-6 is the cleaved species. This raises the possibility that the staining patterns seen in vivo may be those of residual, cleaved, pro-DVR-6 protein, and not full-length protein. At present, the resolution of immunostained sections by light microscopy does not allow us to distinguish whether this protein is entirely intracellular, or whether the cleaved proregion also accumulates in the extracellular environment.
Additionally, the persistence of cleaved pro-DVR-6 in steady-state protein suggests that it may have a function of its own.

One important conclusion reached using cDVR-6 is that only a subset of tissues previously shown to express DVR-6 mRNA express the protein. The PYS cell line (which expresses DVR-6 transcripts but not protein) should provide clues as to the mechanism(s) involved in this discrepancy of mRNA and protein expression. Tissues which do express both DVR-6 transcripts and protein in the mouse are epithelial and nervous tissues. While the functional role(s) of this protein is not known at this time, these expression data presented here allow us to begin proposing and testing potential functions. Additionally, cDVR-6 should be useful as an early marker for neuronal differentiation in the developing nervous system.

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