Axonal localisation of the CAM-like tyrosine phosphatase CRYPα: a signalling molecule of embryonic growth cones

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SUMMARY

Migrating embryonic growth cones require multiple, membrane-associated signalling molecules to monitor and respond to guidance cues. Here we present the first evidence that vertebrate cell adhesion molecule-like protein tyrosine phosphatases are likely to be components of this signalling system. CRYPα, the gene for an avian cell adhesion molecule-like phosphatase, is strongly expressed in the embryonic nervous system. In this study we have immunolocalised the protein in the early chick embryo and demonstrated its predominant localisation in axons of the central and peripheral nervous systems. This location suggests that the major, early role of the enzyme is in axonal development. In a study of sensory neurites in culture, we furthermore show that this phosphatase localises in migrating growth cones, within both the lamellipodia and filopodia. The dependence of growth cone migration on both cell adhesion and signalling through phosphotyrosine turnover, places the cell adhesion molecule-like CRYPα phosphatase in a position to be a regulator of these processes.

Key words: phosphotyrosine, cell adhesion molecule, axonogenesis, chicken

INTRODUCTION

Cell migration is an essential process in embryonic development, and relies on the cellular recognition of microenvironmental cues to generate and direct cell movement. A good example of this is seen in embryonic axons, whose growth cones migrate to target tissues using guidance cues on axon fascicles, glia and the extracellular matrix. Growing axons therefore represent a valuable model of directed cell movement, and one in which the transmembrane signalling mechanisms can be characterised.

Identification of the signalling molecules that regulate growth cone movement is essential to understanding the biological process. The growth cone can probe its microenvironment using a range of cell surface receptors, including immunoglobulin (Ig) superfamily cell adhesion molecules (CAMs; Jessell, 1988; Grumet, 1991), cadherins (Doherty and Walsh, 1991), integrins (Letourneau et al., 1988), and receptors for growth and differentiation factors. There is increasing evidence that protein tyrosine phosphorylation is a mediator of many intracellular signals downstream from these receptor systems. During axonal adhesion for example, the ligands of CAMs L1 and N-CAM trigger intracellular second messenger cascades which include rapid changes in cellular phosphotyrosine (Schuch et al., 1989; Atashi et al., 1992; Doherty and Walsh, 1992; Williams et al., 1994c). Numerous receptor and non-receptor protein tyrosine kinases (PTKs) are located in nerve cells (Gertler et al., 1989; Bixby and Jhabvala, 1993; Henkemeyer et al., 1994), and inhibitor studies have directly implicated these enzymes in regulating filopodial movement and growth cone migration (Bixby and Jhabvala, 1992; Miller et al., 1993; Wu and Goldberg, 1993; Ignelzi et al., 1994; Williams et al., 1994c). A specific interaction between vertebrate CAMs and the receptor for fibroblast growth factor, which is a receptor PTK (R-PTK), has recently been demonstrated to play a role in axon outgrowth (Williams et al., 1994a,b). Links between PTK signalling and growth cone pathfinding are also found in Drosophila, where the co-inactivation of the abl PTK gene and the CAM gene fasciclin I causes aberrant guidance of pioneer axons (Elkins et al., 1990).

Growth cone movement away from repulsive cues has also been linked to the activity of PTKs in cultured neurons (Finnegan et al., 1993; Smallheiser, 1993).

Embryonic cells, and in particular nerve cells, can control their movement therefore through the combined use of cell surface CAMs and both receptor and non-receptor PTKs. The search for axonal components which balance the action of PTKs has recently focused attention on protein tyrosine phosphatases (PTPs). There are now many members in this gene family, including a growing number of receptor-like PTPs (R-PTPs). Significantly, some of these are extracellularly related to Ig superfamily neural CAMs (Streuli et al., 1989; Tian et al., 1991; Canoll et al., 1993; Jiang et al., 1993; Levy et al., 1993; Mizuno et al., 1993; Sahin and Hockfield, 1993; Walton et al., 1993; Yan et al., 1993; Stoker, 1994), and several of these R-PTP genes are expressed in neural tissues of adult and late fetal mammals. Furthermore, expression of R-PTP genes is also found in the early embryonic central nervous system.
(CNS) of invertebrates (Tian et al., 1991; Yang et al., 1991), and we have recently identified a family member, encoded by the CRYPα gene, whose RNA is expressed strongly in the early embryonic nervous system of the chick (Stoker, 1994). These findings suggest that CAM-like R-PTPs play conserved roles alongside PTKs in the embryonic nervous system during certain stages of axonogenesis. One logical role of such enzymes would be to serve as direct links between the adhesive microenvironment and phosphotyrosine signalling in these axons.

Although the ligands for CAM-like R-PTPs in vivo are not known, two phosphatases have been shown to exhibit homophilic adhesive interactions in cultured (non-neural) cells (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994). The downstream targets of R-PTP catalytic activity include PTKs of the src family which can be activated through dephosphorylating carboxy-terminal tyrosine residues (Cooper and King, 1986; Mustelin et al., 1989; Zheng et al., 1992; Burns et al., 1994; Fang et al., 1994). Given that such PTKs play a part in axonogenesis and have cytoskeletal targets such as tubulin (Matten et al., 1990), the colocalising neural R-PTPs would also be implicated in axon growth control.

In this study we have determined the embryonic distribution of the CAM-like R-PTP encoded by the avian CRYPα gene. We demonstrate that CRYPα proteins reside predominantly in the developing axonal network of the central and peripheral nervous systems (CNS and PNS). Moreover, in a study of cultured neurons the protein has also been located in the growth cones of neurites. These are proposed to be the important functional locations for the protein in early development, and the implications of this are discussed.

MATERIALS AND METHODS

Generation of antigens for antisera production

Sera RP1 and IG2 were raised against fusion proteins synthesised in bacteria. Segments of the CRYPα cDNA were isolated either using restriction enzyme sites, or polymerase chain reaction using Pfu enzyme (Promega). The region encoding amino acids 397-836 was subcloned into the 

Xho

I restriction enzyme site of pET20b

Eco

RI site of pET20b

sequence, GenBank no. 32780; Stoker, 1994). The open reading frames were fused to poly-histidine

sequences for affinity purification. The proteins were synthesised in BL21 LysS bacteria (InVitrogen) after induction with isopropyl-β-D-thiogalactopyranoside for 3 hours, and were affinity purified on a nickel binding column (His-Bind resin, InVitrogen). After confirming protein purity on polyacrylamide gels, the proteins were injected with TiterMax adjuvant (VaxcelTM, Inc, GA, USA) into young adult Dutch rabbits. Immunoglobulin was obtained from sera using ammonium sulphate or sodium sulphate precipitation methods.

Western immunoblotting

Tissues were lysed in RIPA buffer (0.1% SDS, 1% NP40, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris HCl, pH8.0) containing 5 μg/ml aprotinin. Ten to 20 μg of tissue protein was separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Schleicher & Schuell) and treated with the diluted sera (1:200 for IG2; 1:1000 for RP1). Bound antibody was visualised with anti-rabbit alkaline phosphatase secondary antibody (Promega). To block antibody with antigen, 2.5-5 μg of purified fusion protein was pre-mixed with each 10 μl of serum for 1 hour, before dilution of the serum and addition to the filter.

Immunohistochemistry and immunofluorescence

Embryos were fixed in 4% paraformaldehyde in PBS for 1-2 hours on ice, then equilibrated to 15% sucrose in PBS overnight. Tissue was embedded in OCT compound (Tissue-Tek) and sectioned at 5-10 μm and mounted on gelatin-coated glass slides.

For immunofluorescence and immunoperoxidase analysis, the tissue sections were pre-treated with PBS/1% bovine serum albumin (fraction V)/0.25% Triton X-100, for 10 minutes, rinsed in PBS/0.1% BSA, and the primary antibody applied in PBS/1%BSA. IG2 was used at dilutions of 1:500 to 1:1000, and RP1 at 1:1000 to 1:2000. Primary antibody was removed after 1 hour, the slides rinsed twice for 5 minutes in PBS/0.1% BSA, before addition of secondary antibodies. Secondaries were pre-incubated for 30 minutes in 5% chick serum/PBS, added to the sections for 30 minutes, then sections were washed twice for 5 minutes in PBS/0.1% BSA. The antibody 3A10 detects neurofilament-associated components in all early axons (Developmental Studies Hybridoma Bank; Yamada et al., 1991). Secondary antibodies were: anti-rabbit FITC (Sigma); horseradish peroxidase (HRP)-conjugated anti-rabbit (Promega); HRP-conjugated anti-mouse (DAKO). HRP was visualised with diaminobenzidine using standard methods. Photomicrographs were taken with a Leitz DM7 microscope.

Tissue culture

E7 DRG were isolated and dissociated in 0.1% trypsin in Dulbecco’s Modified Eagle’s Medium (DMEM) at 37°C for 30 minutes. Cells were pelleted at 3000 rpm for 2 minutes, and resuspended in growth medium: DMEM, 1% chick serum, 0.2% low serum protein replacement (Sigma), 50 μg/ml gentamycin, 10 ng/ml nerve growth factor (NGF; gift from A. Tolkovsky, University of Cambridge). The cells were preplated on bacteriological plates for 2 hours at 37°C to remove adherent, non-neural cells. The non-adherent, predominantly neural cells were counted and plated onto coated glass coverslips (poly-L-lysine at 20 μg/ml for 30 minutes, followed by laminin at 45 μg/ml for 30 minutes, then air dried). Cells were maintained in growth medium at 37°C. After 20 hours the cells were fixed in 2% paraformaldehyde plus 15% sucrose at room temperature for 30 minutes. Cells on coverslips were processed for antibody binding as described above except that all washes were performed in PBS/0.1% BSA/0.25% Triton X-100, and primary antibodies RP1 and the preimmune were used at dilutions of 1:100 to 1:200.

Cell transfection

The CRYPα cDNA was subcloned into the plasmid pRc/RSV (InVitrogen) as an XbaI fragment, placing it under the transcriptional control of a Rous sarcoma virus promoter. Quail QT6 cells (Moscovici et al., 1977) were transfected with 15 μg plasmid DNA using the calcium phosphate co-precipitation method ( Graham and Eb, 1973). Cells were lysed in RIPA buffer after 20 hours, and lysates subjected to immunoblotting as above.

RESULTS

Identification of embryonic CRYPα proteins

CRYPα mRNA is expressed strongly in embryonic neural tissues, and two major isoforms of the protein are predicted to be expressed from alternatively spliced transcripts (Stoker, 1994). Two antisera were raised against fusion proteins containing parts of the CRYPα extracellular domain, and each antiserum should detect both CRYPα isoforms (see Materials and Methods). These antibodies were used first to identify the
phosphatase protein in immunoblots of embryonic brain tissue. Proteins of 150×10^3 and 90×10^3 $M_r$ were detected specifically with both antisera, although antiserum RP1 was apparently more sensitive than IG2 (Fig. 1A). The preimmune sera did not recognise these proteins, and the reactivity of immune sera could be blocked with purified antigens (Fig. 1A). The identity of the strong IG2 preimmune band at 58×10^3 $M_r$ is not known; the preimmune serum does not show recognisable specificity in immunohistochemical staining of tissue sections (see below).

The predicted relative molecular masses of the major isoforms CRYP$\alpha_1$ and CRYP$\alpha_2$ are 150×10^3 and 205×10^3 respectively (Stoker, 1994). Given that the closely related RPTP encoded by the LAR gene (approximately 200×10^3 $M_r$ in size) is cleaved to give a 150×10^3 $M_r$ protein (Streuli et al., 1992; Yu et al., 1992; SerraPages et al., 1994), our data suggest that both CRYP$\alpha$ proteins are similarly cleaved to give the 150 and 90×10^3 $M_r$ species (Fig. 2; see Discussion). To support this, and to confirm the specificity of the antisera, an expression plasmid containing the cDNA for the smaller CRYP$\alpha$ isoform was transfected into cultured QT6 cells, which do not themselves express high levels of this protein. In cells transfected with the sense construct, but not the antisense construct, the predicted 90×10^3 $M_r$ protein was observed (Fig. 1B). In addition, the sense-transfected cells reacted specifically with the antisera in immunocytochemical studies (not shown).

A further protein of approximately 60×10^3 $M_r$ was reproducibly observed in the IG2 immune, but not preimmune, immunoblots (Fig. 1A, small arrow), running just above the strong, non-CRYP$\alpha$ related preimmune band. This 60×10^3 $M_r$ protein is not detected by the RP1 serum, and its identity is presently unclear. Two possibilities are that it represents a further processed form of the CRYP$\alpha$ extracellular domain, or a novel CRYP$\alpha$ isoform.

**Developmental regulation of CRYP$\alpha$ isoform expression**

The CRYP$\alpha$ gene produces two major transcripts in the
embryo brain (Stoker, 1994), which are likely to encode for the 90×10^3 M_r and 150×10^3 M_r proteins expressed at E16. The differential RNA splicing which produces these transcripts is developmentally regulated in the brain (Stoker, 1994). To examine whether or not this is reflected in the protein expression, brain tissues from E4 to E16 were examined (Fig. 1C). The 150×10^3 M_r protein was expressed throughout this period of development with a modest but reproducible peak at E6. The 90×10^3 M_r protein was not expressed until after E4 in the brain (Fig. 1C), rising to a plateau at E10 and remaining constant thereafter. This pattern is broadly consistent with the RNA data (Stoker, 1994). The 90×10^3 M_r protein was also observed in E16 spinal cord, but not in other non-neural E16 tissues (data not shown). CRYPα proteins present in the brain therefore show developmental regulation prior to E10, and the smaller CRYPα1 isoform is specifically concentrated in the nervous system.

Localisation of CRYPα proteins in the embryo

To determine the tissue distribution of CRYPα proteins during early neural development, immunohistochemistry was carried out on cryostat sections of E3 and E4 embryos. These early stages were of interest because of the high rates of neural differentiation and axonogenesis which are occurring in both the CNS and PNS.

Localisation at E3

Transverse sections taken at the forelimb level of the trunk were examined using the two polyclonal antisera. The IG2 antiserum detected the phosphatase prominently in axons of the embryo, co-localising with the neurofilament-associated antigen 3A10 seen in serial sections (Fig. 3A,B). RP1 showed a similar specific reactivity (Fig. 3C,D). Most of the different CNS nerve fibre types in the trunk contained the protein, including those within the segmental nerve roots (Figs 3, 4B,
Fig. 4. CRYPα protein localised in spinal axons and dorsal root ganglia at E3. E3 embryos were sectioned at the forelimb level and CRYPα detected using antibodies IG2 (B,D), and RP1 (C,F). Axons were located in serial sections using neurofilament-associated antigen 3A10 (A,E). CRYPα is abundant in the ventral root in early motor axons (arrows in B and C) and in DRG axons (arrowhead in C; with 3A10 antibody in A, arrowhead). The protein is also seen in lateral axons (arrowheads in B and F) and in the early axons of the ventral commissure (open arrows in D,E,F). Scale bars: 50 μm.
4C), the ventral commissure (Fig. 4D,E,F), and lateral spinal cord axons (Fig. 4B,F). Little of the phosphatase was detected within the neuroepithelium or the differentiated neurons of the spinal cord. Outside of the nervous system, weak but specific immunoreactivity of CRYPα was detected in connective tissues, in particular within gut mesenchyme (not shown). Little if any specific expression was seen in epithelial cells of for example the epidermis or gut.

Both antisera gave similar immunoreactivity in the spinal cord. It was noted, however, that early DRG axons were not detected using the IG2 serum (Fig. 4B), whereas RP1 was able to detect at least a proportion of these sensory axons (Fig. 4C). This may be partly explained by RP1 having slightly greater avidity than IG2. Nevertheless, since regions such as the ventral root axons reacted similarly with both sera, we believe that the differential DRG axon reactivity has some basis in specific antigen localisation. Since the two antisera were raised against different extracellular regions of CRYPα, it is possible that they are detecting distinct protein isoforms in certain axon populations (see Discussion).

**Localisation at E4**

The predominant expression of CRYPα proteins in the nervous system is more evident at E4. In the trunk, expression followed broadly the pattern of axons detected with 3A10 (Figs 5, 6), with the phosphatase being found at high levels in segmental nerves, commissural axons, axons of the ventral and dorsal funiculi, and DRG axons (Figs 5, 6). This is consistent with CRYPα mRNA expression at E4, seen in cells of the spinal cord and DRG (Stoker, 1994). As at E3, CRYPα protein

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**Fig. 5.** Localisation of CRYPα at E4. Transverse sections through the forelimb level of E4 embryos, immunohistochemically stained with anti-CRYPα sera IG2 (A) and RP1 (C), and antibody to neurofilament-associated antigen 3A10 (B,D). CRYPα is abundant in axons of the spinal cord (asterisk in B and D), ventral nerve roots (open arrowheads in B and D) entering the limbs (triangles in A), DRG (horizontal arrow in D), ventral commissure (vertical arrow in B and D), and dorsal funiculus (angled arrow in B and D). Weaker staining is also seen in the spinal cord, connective tissues, and the myotome (arrow in C). B and D are serial sections of A and C, respectively. Scale bar: 1 mm.
localised prominently in axons, with relatively little around the cell bodies of the spinal cord neuroepithelium or the spinal and DRG neurons. Whereas RP1 and IG2 broadly shared patterns of reactivity at E4, there were again some reproducible differences. First, as at E3, nerve processes within the DRGs and dorsal roots stained more clearly with RP1 (Fig. 6A) than with IG2 (Fig. 6C). However, at this stage both sera reacted with the clearly defined dorsal funiculus which also contains predominantly DRG-derived fibres. The differential detection of fibres in the DRG itself may therefore be partly explained by greater RP1 avidity. However, this does not easily explain the second example of differential reactivity which occurred in the ventral commissure of the spinal cord. This commissure stained significantly more strongly with IG2 than with RP1 (Figs 5A,C, 6A,C). With IG2 the commissure reacted more strongly than the ventral funiculi (Fig. 5A); a situation not found either with RP1 or 3A10 (Fig. 5B-D). Given that IG2 is also probably the weaker of the two polyclonal antisera, this stronger commissural signal is likely to represent a real increase in the amount of antigen at this site.

In cranial regions CRYPα was present at high levels in peripheral nerves such as the oculomotor nerve (Fig. 7C), and in cranial ganglia such as the facioacoustic ganglion (Fig. 7A,B). Strong localisation was also seen in nerve fibre layers of the brain (Fig. 7C).

At E4 there was a low level of staining for CRYPα in connective tissues throughout the embryo. Using the RP1 antiserum, an elevation in signal was also often detected in trunk myotome (Fig. 5A). No significant level of CRYPα protein was evident in epithelia of the gut, skin, or primitive kidney (Figs 5, 6).

In these studies the preimmune sera did not show specific reactivity, and the reactivity of immune sera could be blocked by preincubation with the fusion protein immunogens (Fig. 6D).

**Location of CRYPα in growth cones**

The predominant location of CRYPα protein in the early embryo is in the developing axonal network. To determine whether or not this protein localised to the growing tips of these axons, a study was made of motile growth cones of cultured neurons. An immunofluorescence study was performed with neurons taken from E7 DRGs. In these neurons, CRYPα was detected along regenerating neurites, and furthermore CRYPα protein was also distinctly localised within growth cones (Fig. 8). The protein was observed both in the lamellipodia and filopodia of these structures (Fig. 8A-C). The growth cone location of the protein strongly suggests that the enzyme serves to regulate phosphotyrosine in these motile structures.

Of further interest was the heterogeneity of CRYPα expression in the E7 DRG neurite population. Although most neurites contained significant amounts of CRYPα protein, a range of strong to weak staining intensities was evident (Fig. 8F). No obvious correlation could be seen between neurite length and CRYPα level on the laminin substrate, suggesting that laminin-mediated axon growth can proceed irrespective of CRYPα expression level in this culture system. CRYPα has also been observed in growth cones grown on poly-L-lysine alone, again at variable expression levels (not shown). The heterogeneity in CRYPα staining may be due in part to the presence in culture of different E7 DRG neuron types, and this warrants further study.

**DISCUSSION**

The control of growth cone migration and nerve formation in the embryo depends on the action of cell surface-associated molecules acting in concert to detect and respond to microenvironmental cues. These molecules include CAMs, integrins, and several receptor and non-receptor tyrosine kinases. In this report we have presented the first evidence that a CAM-like R-PTP enzyme is also a prominent component of developing axons in the vertebrate embryo.

The CRYPα phosphatase is expressed in a wide variety of axon types in the early chick embryo, both within the CNS and the PNS. The function of the molecule may therefore be quite general in early axons. Alternatively, whereas its own expression is relatively broad, the activity of CRYPα may depend on a ligand(s) which has a more restricted spatial distribution. The phosphatase is expressed in some of the earliest embryonic axons which are generated in the spinal cord, including those from the early motor neurons, and it is also seen in early sensory axons. Therefore it is likely that the function of the protein is first required during phases of rapid growth and fasciculation of these axons, before synapse formation has started. Later in development CRYPα proteins are maintained at high levels in the brain, indicating that the protein may have later functions unrelated to axonogenesis during maturation of nervous tissue.

In rapidly growing axons it would be logical for a CAM-like R-PTP such as CRYPα to be located in the motile structure of the growth cone. It is here that the molecules that detect and respond to growth and guidance cues such as CAMs and PTKs are concentrated. There would be a clear requirement for phosphatase activity in these structures, if not least to create a dynamic counterbalance to the PTK signals. Recent findings have demonstrated an enrichment of such phosphatase activity in growth cone preparations (Bixby and Jhabvala, 1993). Our finding that a vertebrate CAM-like PTP localises within embryonic axons and growth cones is the first direct confirmation that such molecules are located appropriately. CAM-like R-PTPs have also been found in invertebrate CNS axons (Tian et al., 1991; Yang et al., 1991). CRYPα could be either antagonising the action of PTKs or, given the ability of R-PTPs to activate src-family PTKs (Mustelin et al., 1989; Den et al., 1993), CRYPα could be having an agonistic effect on src-related PTKs of axons. Given the proposed role of PTKs in axonal growth, guidance, and retraction (Gertler et al., 1989; Bixby and Jhabvala, 1993; Finnegan et al., 1993; Miller et al., 1993; Smallheiser, 1993; Ignelzi et al., 1994; Williams et al., 1994), it is not difficult to place this phosphatase into a regulatory scenario. As well as PTKs as potential axonal targets, one cytoskeletal target of phosphatases may be tubulin, the microtubule protein which is phosphorylated by pp60c-src under the influence of CAMs (Matten et al., 1990; Atashi et al., 1992). Other potential targets present in growth cones include vinculin and talin (Letourneau and Shattuck, 1989), both of which can be tyrosine phosphorylated in neural and non-neural cells (Igarashi et al., 1990; Tidball and Spencer, 1993). Like tubulin, these molecules are involved in cytoskeletal dynamics and cell movement.

CRYPα protein is also found in axon fascicles and cultured neurite processes. This may in part reflect transport of enzyme to or from its site of activity at the growth cone. Nevertheless,
it is conceivable that this enzyme also has a role in monitoring the interactions of axon membranes either with other axons or with the glia and extracellular matrix in the nerve path. The functional consequences of such an activity remain to be determined, but an effect on the dynamic axonal cytoskeleton, in particular tubulin phosphorylation, is a possibility.

The extracellular ligands of CAM-like R-PTPs in vivo are not known at present, although in cell culture the phosphatases PTPμ and PTPν can promote adhesion through homophilic interactions (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994). Heterophilic interactions between CAM-like R-PTPs and neural Ig superfamily CAMs would also be an attractive possibility, providing a functional link between neural CAMs and for example the activation of downstream src-like PTKs (Atashi et al., 1992; Williams et al., 1994c). The localisation of CRYPα in the early spinal cord and DRGs overlaps with the known distributions of several CAMs, and the growth cone location of these CAMs also coincides with CRYPα (Daniloff et al., 1986; Letourneau and Shattuck, 1989; Krushel et al., 1993; Halfter et al., 1994; Stoker, unpublished data). The possibility of direct interactions either in cis or in trans, therefore exists.

We have found that two isoforms of CRYPα protein which differ in their CAM-like domains are expressed in the developing nervous system, and that their relative expression levels change in the brain as it matures. The interactions of extracellular ligands with CRYPα1 and CRYPα2 may be distinct and could alter the specific role of each protein isoform (discussed also by Stoker, 1994). The extracellular domain of CRYPα1, which appears after E4 in the brain, has not been detected in non-neural tissues (Stoker and Gehrig, unpublished data), indicating that this isoform could serve a neural-
specific function. At and prior to E4 the larger protein isoform CRYPα2, which is expressed in the brain, is likely to represent the majority of the protein detected immunohistochemically at these stages. It cannot be ruled out, however, that further isoforms occur in specific parts of the nervous system. Indeed, the fact that our two antisera react differently to axons of DRG and the ventral commissure may indicate that further, novel isoforms of CRYPα are present. For example, we have previously identified a CRYPα cDNA which would encode only the amino-terminal two Ig domains (CRYPα4; Stoker, 1994), and, conversely, an isoform lacking precisely these two domains has been predicted from the closely related murine phosphatase PTPδ (Mizuno et al., 1993). Since the IG2 antiserum was raised against precisely these two Ig domains, the above protein isoforms would be distinguishable with our antisera. Attempts to identify these putative isoforms are in progress. The immunoblot data also show that the IG2 antiserum detects a 60×10^3 M_r protein in immunoblots which RP1 does not. Although this protein does not correspond to any currently known R-PTP isoform, further variants may well be identified as has been the case recently for LAR (Ogrady et al., 1994). Although the two antisera could have some minor cross-reactivity with related R-PTPs such as LAR and PTPδ, it is considered unlikely; this cannot be ruled out, however, until the location of these other R-PTPs is documented.

The immunoblotting data revealed that CRYPα proteins are post-translationally cleaved in embryonic neural tissues, in a similar manner apparently to that of the phosphatase LAR (Streuli et al., 1992). As recently suggested, the cleavage process could be important in regulating the activity of membrane-anchored PTP enzymes (Streuli et al., 1992; Serra-Pages et al., 1994). It will be of interest to determine also if CRYPα extracellular domains are shed from the axonal membrane, as has been demonstrated for LAR in cultured non-neural cells (Streuli et al., 1992). In embryonic neural tissue the shed domains could act as matrix-associated ligands, or competitive ligands, for axonal receptors.

Finally, some of the neurites grown on laminin as an artificial substrate were seen to contain relatively little CRYPα protein. Laminin-mediated neurite outgrowth, which operates through integrin receptors, therefore may proceed independently of CRYPα level. In vivo, however, a difference in CRYPα level between axons may result in differential growth of axons in the presence of the physiological CRYPα ligand(s). Axons expressing relatively low levels of CRYPα, if present in vivo, may rely instead on related R-PTPs such as LAR or PTPδ. It will be of significant interest therefore to assess the axonal expression of these other R-PTPs.

In conclusion, the prominent axonal location of CRYPα proteins in the embryonic chick indicates the probable major functional site for this CAM-like phosphatase in early devel-
opment. Location of the enzyme in growth cones, whose movement is controlled by adhesion and phosphotyrosine signalling, leads us to propose that CRYP\(\alpha\) is fundamentally involved in these processes.

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**Fig. 8.** CRYP\(\alpha\) protein localised in the neurites and growth cones of cultured DRG neurons. DRG neurons from E7 embryos were cultured on poly-L-lysine and laminin and immunostained with RP1 serum (A,B,C,F) or RP1 preimmune serum (D). CRYP\(\alpha\) is seen in neurite processes and in growth cones. Both the lamellipodia and filopodia (arrows in A,B,C) of growth cones contain the protein. Specific reactivity is seen using preimmune serum in growth cones (arrow in D); the axon of this growth cone runs from the cell body indicated (arrowhead in D). (E) Phase contrast and (F) indirect immunofluorescence images showing the heterogeneity of RP1 reactivity in different neurites. Strongly reactive growth cone (arrowheads), weakly reactive growth cone (large arrow), and segments of weakly reactive neurites (small arrows in F), are indicated. Scale bars: 20 \(\mu\)m.

**REFERENCES**


binding of PTPla, a receptor-type protein tyrosine phosphatase, can mediate cell-cell aggregation. J. Cell Biol. 122, 961-972.


Control of cell pattern in the developing nervous system: Polarizing activity of the floor plate and notochord. *Cell* 64, 635-647.


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