Developmental expression of G proteins in a migratory population of embryonic neurons

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SUMMARY

Directed neuronal migration contributes to the formation of many developing systems, but the molecular mechanisms that control the migratory process are still poorly understood. We have examined the role of heterotrimeric G proteins (guanyl nucleotide binding proteins) in regulating the migratory behavior of embryonic neurons in the enteric nervous system of the moth, Manduca sexta. During the formation of the enteric nervous system, a group of approx. 300 enteric neurons (the EP cells) participate in a precise migratory sequence, during which the undifferentiated cells populate a branching nerve plexus that lies superficially on the visceral musculature. Once migration is complete, the cells then acquire a variety of position-specific neuronal phenotypes. Using affinity-purified antisera against different G protein subtypes, we found no apparent staining for any G protein in the EP cells prior to their migration. Coincident with the onset of migration, however, the EP cells commenced the expression of one particular G protein, Goα. The intensity of immunostaining continued to increase as migration progressed, with Goα immunoreactivity being detectable in the leading processes of the neurons as well as their somata. The identity of the Goα-related proteins was confirmed by protein immunoblot analysis and by comparison with previously described forms of Goα from Drosophila. When cultured embryos were treated briefly with aluminium fluoride, a compound known to stimulate the activity of heterotrimeric G proteins, both EP cell migration and process outgrowth were inhibited. The effects of aluminium fluoride were potentiated by alpha toxin, a pore-forming compound that by itself caused no significant perturbations of migration. In preliminary experiments, intracellular injections of the non-hydrolyzable nucleotide GTPγS also inhibited the migration of individual EP cells, supporting the hypothesis that G proteins play a key role in the control of neuronal motility in this system. In addition, once migration was complete, the expression of Goα-related proteins in the EP cells underwent a subsequent phase of regulation, so that only certain phenotypic classes among the differentiated EP cells retained detectable levels of Goα immunoreactivity. Thus Go may perform multiple functions within the same population of migratory neurons in the course of embryonic development.

Key words: cell migration, G protein, neuronal motility, migratory pathway, enteric nervous system, invertebrate embryogenesis, Manduca sexta

INTRODUCTION

The directed migration of neurons or their undifferentiated precursors is a prominent feature in the developing nervous systems of many organisms. Besides distributing the cellular components of a particular region to their appropriate locations, the process of migration exposes immature neurons to a variety of epigenetic cues, thereby influencing the morphological and biochemical phenotypes that they may subsequently express (e.g. Black et al., 1984; Teillet et al., 1987; Walsh and Cepko, 1988; Wets et al., 1989). At the level of an individual neuron, several distinct extracellular and intracellular events must be coordinated so that migration can proceed normally. The neuron must recognize appropriate cues for orientation, presumably via specific receptor subtypes, which must be expressed at the requisite stages of development. These ligand-sensitive receptors must be coupled to one or more intracellular signalling systems within the cell, which in turn must be competent to transduce appropriate stimuli to one or more effector systems that are associated with the motile apparatus of the cell. Ultimately, this sequence must result in regulated changes in the cytoskeleton (Singer and Kupfer, 1986; Smith, 1988; Theriot and Mitchison, 1992), although other cellular processes may also be affected, including modifications in the secretory apparatus, reorganization of membrane-limited receptors, altered rates of organelle transport, and specific changes in gene expression (reviewed by Singer and Kupfer, 1986; Caterina and Devreotes, 1991). Beyond a simple activation of the locomotory process, directed migration also requires that continued input from environmen-
tal cues be provided, so as to prevent the uncontrolled dispersal of neurons (or other cell types) into inappropriate domains of the developing organism (e.g. Barth, 1987; Grimstad, 1987). Among the intracellular signalling systems that may affect motility, the heterotrimeric G proteins (guanyl-nucleotide binding proteins) have been implicated in a number of different studies of both cell migration and process outgrowth. The G proteins comprise a conserved family of molecules that are involved in transmembrane signal transduction in virtually all eukaryotic cells (Bourne et al., 1986; Bourne et al., 1991; but see also Federman et al., 1992; Kleuss et al., 1992). Several different classes of G proteins have been identified in the developing nervous systems of both vertebrates (Pituello et al., 1991; Rius et al., 1991; Shinozaka et al., 1992) and invertebrates (Wolfgang et al., 1990; Ray and Ganguly, 1992). In particular, members of the Go class have been associated with regions of active outgrowth and presumed synaptogenesis (Chang et al., 1988; Garibay et al., 1991; Wolfgang et al., 1991; Asano et al., 1992).

Go-related proteins have been shown to form a major component of growth cone-like processes in PC-12 cells (Strittmatter et al., 1990; Edmond et al., 1990; Garibay et al., 1991), and several different adhesion molecules stimulate process outgrowth in these cells via one or more permissiveness-sensitive mechanisms (which might involve Go; Schuch et al., 1989; Doherty et al., 1991). Recently, the inhibitory response of neuronal growth cones to several different collapsing factors has also been shown to be G protein-dependent (Igarashi et al., 1993), indicating that G protein-coupled mechanisms may exert a negative regulation on neuronal motility, as well. G proteins have also been implicated in the chemotactic responses of non-neural cells, including polymorphonuclear leukocytes and cells of the slime mold Dictyostelium (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991). The mechanisms by which G proteins affect the locomotory process in these systems remain enigmatic, however. In the case of neuronal migration, the role of particular G proteins in regulating motility has not been examined.

To address this issue, we have begun an investigation of neuronal migration in a relatively simple preparation, the enteric nervous system (ENS) of the moth, Manduca sexta. As previously described, the ENS of Manduca includes a pair of small peripheral ganglia and a distributed population of ~300 enteric neurons (the EP cells) that occupy a branching nerve plexus, called the enteric plexus (Copenhaver and Taghert, 1989a,b; see Fig. 1A). The EP cells are noteworthy in that the entire population shares a common developmental origin, emerging from an epithelial placode in the foregut between 30-40% of embryogenesis (Copenhaver and Taghert, 1990). Immediately thereafter, the EP cells undergo two successive waves of directed migration, first spreading down both sides of the foregut-midgut boundary (from 40-55% of development) and then rapidly dispersing along a defined set of muscle bands that have recently differentiated from the visceral musculature (55-60%). It is during this second migratory phase that the mature distributions of the EP cells are established. Only after migration is complete do the neurons commence the expression of their mature phenotypes, at which time they exhibit a number of distinct characteristics in a position-specific manner. For example, several distinct morphological subtypes have been identified that correspond to the terminal locations occupied by the EP cells on either the foregut or midgut (Copenhaver and Taghert, 1989b). In addition, one particular subtype (designated ‘type B’) has been shown to commence the expression of neuropeptides related to the molluscan peptide Phe-Met-Arg-Phe-NH₂ (FMRFamide), but only after the neurons have completed their migration onto the midgut (Copenhaver and Taghert, 1989a). Both the migration and differentiation of the EP cells have been shown to proceed in embryo culture (Copenhaver and Taghert, 1990; Copenhaver, 1993), facilitating an investigation of the migratory process in a normal developmental context.

We now report that neuronal migration in this system coincides with the expression of a particular G protein that may in turn regulate key aspects of migratory behavior. Specifically, we have found that the EP cells commence the expression of a protein related to Go₄ just prior to the onset of their migratory dispersal, and the levels of expression subsequently increase in all of the neurons throughout the period of active migration. Once migration is complete, however, the pattern of Go₄ expression in the enteric plexus undergoes an additional phase of regulation, in that only certain subsets of the EP cells continue to exhibit detectable levels of Go-related protein. This subsequent pattern of expression is directly correlated with the mature phenotypes that the neurons acquire during the maturation of the ENS. In addition, stimulating G protein activity in the EP cells with either a brief exposure to aluminium fluoride (AlF₄⁻) or by injection of the non-hydrolyzable nucleotide GTPγS, significantly inhibits both their migratory dispersal and subsequent axonal outgrowth. Thus, in this system, the developmental expression of Go₄ is precisely regulated with respect to the onset of neuronal migration and may play an important role in controlling subsequent aspects of the migratory process. A preliminary account of some of these results has appeared in abstract form (Copenhaver et al., 1992).

**MATERIALS AND METHODS**

**Animal preparation**

A laboratory colony of Manduca sexta was used to generate experimental animals, as previously described (Copenhaver and Taghert, 1989a). Eggs were collected at 1 hour intervals as needed and maintained in a 25°C incubator. At this temperature, embryogenesis is completed in ~100 hours (1 hour is equivalent to 1% of development). Established schedules of external and internal markers (Dorn et al., 1987; Copenhaver and Taghert, 1989a) were used to identify specific developmental stages. Embryos were removed from their shells and underlying membranes and dissected along the dorsal body wall to expose the ENS in the following medium (after Chen and Levi-Montalcini, 1969; and Seecof et al., 1971): 50% Schneider’s Drosophila medium, 40% minimal essential medium (MEM) with Hanks’ salts, 9.7% heat-inactivated fetal calf serum, 0.2% edible, 0.1% insulin, 0.01% penicillin-streptomycin. Embryo cultures were also supplemented with Manduca hemolymph (5-10%, vol/vol; Copenhaver and
Taghert, 1990; Copenhaver, 1993). Under these conditions, semi-intact embryos can be cultured for up to 50% of subsequent development (Copenhaver and Taghert, 1990, 1991). For postembryonic stages, animals were dissected in a modified Weever’s saline (in mM: dextrose, 160; KCl, 40; MgCl2, 18; NaCl, 4; CaCl2, 3; KH2PO4, 1.25; KHCO3, 1.25; pH 6.5; after Levine and Truman, 1985). Animals to be processed for immunohistochemical staining were usually fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour at room temperature. Alternatively, some animals were fixed in PLP (2% paraformaldehyde, 10 mM NaIO4, 75 mM lysine, 37 mM phosphate buffer, pH 6.5; after McLean and Nakane, 1974) for 2 hours, which enhanced the detection of Goα when visualized by immunofluorescence.

**Immunohistochemistry**

Fixed preparations were rinsed in PBS and incubated overnight at 4°C in an appropriate dilution of primary antibody. An affinity-purified antiserum generated against the conserved carboxy-terminal decapentapeptide of the α-subunit of Go (Goldsmith et al., 1987; gift of Drs M. Forte and W. Wolfgang) was used at a concentration of 1:10,000 in PBS with 0.1% Triton X-100, 0.1% NaN3, and 10% normal serum. The specificity of this antiserum has been previously characterized in Drosophila (Thambi et al., 1989), in which it recognizes a single protein species (Mr ≈40×103) that has been identified as Goα. The antiserum is completely inhibited when preadsorbed against the carboxy-terminal decapentapeptide of Goα, but it shows no cross-reactivity with other known Gα subunits (Thambi et al., 1989; Wolfgang et al., 1990). An antiserum specific for the α-subunit of Gs (designated ‘RM’; Wolfgang et al., 1990) was also used at a dilution of 1:6000. The monoclonal antibody TN-1 was used as a histological marker for the EP cells, at a concentration of 1:20,000. TN-1 recognizes a cell surface molecule that is related to fasciclin II (Nardi, 1990, 1992) and that is expressed by specific subsets of neural and non-neural cells in Manduca (Taghert et al., 1986; Carr and Taghert, 1988). As previously reported, TN-1 provides an unambiguous marker for the EP cells and their processes throughout embryonic development (Copenhaver and Taghert, 1989b, 1990). To characterize the expression of peptidergic phenotypes in the ENS, a polyclonal antiserum characterized in Drosophila was used at concentrations of 1:100, 1000 (Copenhaver and Taghert, 1989a). For double-labeling experiments, the anti-Goα antiserum was applied in conjunction with a monoclonal antibody that was generated against the moluscan Small Cardiac Peptide (SCP; diluted 1:20; gift of Drs B. Masinovsky and A. O. D. Willows), which also stains all FMRFamide-immunoreactive neurons in the ENS of Manduca. As the specific identity of the neuropeptides produced by the EP cells is still in question, these anti-peptide antisera were simply used to distinguish the type B neurons that express this particular peptidergic phenotype.

Following incubation in primary antisera, the tissues were routinely processed using a biotinylated secondary antiserum, followed by avidin-HRP (Vector Laboratories, Inc; Burlingame, CA), and reacted with 1.4 mM dianinobenzidine in 0.001% H2O2. Stained preparations were then reacted in 90% glycerol in PBS plus 0.1% n-propyl gallate as an oxidant. For co-localization experiments, the anti-Goα antiserum was detected with a biotinylated anti-rabbit antiserum followed by avidin-fluorescein, while the anti-SCP antibody was detected with a biotinylated anti-rabbit antiserum. A biotinylated secondary antiserum, followed by reaction with the appropriate enzymatic substrates (Promega; Madison, WI). The specificity of the reaction was monitored by pre-incubation of the anti-Goα antiserum with an excess quantity of the peptide used to generate the antiserum (Goldsmith et al., 1987) and by incubation of identical blots with antisera specific for other G protein α-subunits; no cross-reactivity among these antisera has been detected in insect tissues (see also Wolfgang et al., 1990, 1991).

**Immunoblot analysis**

Pooled embryonic and postembryonic tissues were collected on dry ice and then rapidly homogenized in sample buffer at 100°C. Approximately 100 µg protein from each sample was separated on 10% SDS-polyacrylamide gels. The protein was subsequently blot-transferred to nitrocellulose, which was then incubated with the same anti-Goα antiserum that was used for immunohistochemistry (after Otter et al., 1987). Bound antibodies were then visualized by incubation with an anti-rabbit antiserum coupled to alkaline phosphatase, followed by reaction with the appropriate enzymatic substrates (Promega; Madison, WI). The specificity of the reaction was monitored by pre-incubation of the anti-Goα antiserum with an excess quantity of the peptide used to generate the antiserum (Goldsmith et al., 1987) and by incubation of identical blots with antisera specific for other G protein α-subunits; no cross-reactivity among these antisera has been detected in insect tissues (see also Wolfgang et al., 1990, 1991).

**AlF4− experiments**

Embryos at several different developmental stages around the time of migration were removed from their protective membranes and restrained in a Sylgard (Dow Corning; NY) chamber under culture medium. A small opening was made in the dorsal epithelium to expose the premigratory EP cells. In our initial experiments, the medium was then replaced with medium containing 25-100 µM AlF4− (prepared from fresh stocks of 1 mM AlCl3 and 500 mM NaF in culture medium, combined in a final ratio of 1:2000; after Jones et al., 1990). The embryos were then allowed to develop for an additional 6-8 hours at 28°C, followed by fixation and staining with TN-1. In subsequent experiments, the time of exposure to AlF4− was reduced by application of bacterial alpha toxin (α-hemolysin from Staphylococcus aureus; List Biologicals; Campbell, CA). This compound has been shown to form transient pores in most plasma membranes, rendering cells permeable to small molecules (Mr<4×103) without inducing cell lysis (McEwen and Arion, 1985; Hohnman, 1988). Alpha toxin (0.02-0.01 mg/ml) was applied to dissected embryos in the presence of 25 µM AlF4− for 20-30 minutes, followed by extensive rinsing in normal medium. The preparations were then allowed to continue to develop for an additional 8-10 hours before fixation. Standard controls for these experiments included both dissected and undissected embryos that were incubated in normal medium, or animals that were incubated for 20-30 minutes in either AlF4− or alpha toxin alone. As an additional control, one group of animals was incubated first with AlF4−, rinsed, and then incubated with alpha toxin over the course of 30 minutes. All preparations were subsequently labelled with TN-1 (as described above), and the extent of EP cell migration and process outgrowth was analyzed with respect to matched controls.

**Intracellular pressure injection**

Individual EP cells were injected at specific times during their migration using a WPI pneumatic (‘Picopump’) pressure injector. Staged embryos were dissected to expose the developing ENS and positioned under a water immersion lens on a modified Nikon UM-2 microscope equipped with an orbital stage from Meridian Instruments, Inc. (Kent, WA). Electrodes were pulled from filament glass on a Sutter P-80/PC puller to a resistance of 10-30 MΩ (when filled with 3 M KCl). Electrodes were filled at the tip with a buffered injection solution containing 1-10 mg/ml lysinated tetramethylrhodamine dextran amine (LRD; from Molecular Probes, Eugene, OR) and backfilled with 1.2 M LiCl or 2 M KAC. For some experiments, the injection solution also contained 20 mM GTPγS, a non-hydrolyzable form of GTP (Stryer and Bourne, 1986). Electrodes were positioned using a Nikon/Narishige ‘joystick’ hydraulic manipulator. Penetration of individual cells was monitored using a Getting Model 5A
intracellular amplifier mounted in parallel with the pressure injector. Solutions were injected using a brief series of 100 milliseconds pulses delivered with an ejection port pressure of ~5 psi; the ejection pressure was adjusted so that no detectable swarming of the target cells accompanied an individual pulse. Following each injection, the preparations were briefly examined using a heavily filtered UV light source to verify the number and position of labelled cells. Embryos were then allowed to continue to develop in culture at 28°C for an additional 8-12 hours before fixation and counterstaining with TN-1. Preparations were photographed at 650x, and the images scanned and analyzed using a Microtek ScanMaker and Adobe Photoshop software.

RESULTS

Goα expression during EP cell migration

The postembryonic ENS of Manduca extends the length of the alimentary tract (foregut, midgut and hindgut) and innervates the superficial layers of the visceral musculature. In the vicinity of the foregut-midgut boundary, this innervation is supplied by the enteric plexus (Fig. 1A), a branching network of nerves projecting along discrete sets of muscle fibers on the foregut and along the eight major longitudinal muscle bands of the midgut (L1-L4 and R1-R4). Anteriorly, the enteric plexus is connected with the enteric ganglia of the foregut by the recurrent nerve (RN; Copenhaver and Taghert, 1991), while posteriorly, its nerves extend most of the length of the midgut and supply a diffuse innervation to the midgut musculature (Copenhaver and Taghert, 1989a). Within the enteric plexus is distributed a population of about 300 neurons (the EP cells). Unlike most neurons of the insect CNS, the EP cells do not occupy cell-specific positions but are distributed variably among the different branches of the plexus. As previously illustrated (Copenhaver and Taghert, 1989b), these distributions are achieved by the directed migration of the EP cells into the different domains of the enteric plexus between 55 and 60% of embryonic development (Fig. 1B).

To investigate whether G proteins may be involved in regulating the motile behavior of the EP cells, the developmental expression of different G proteins in the embryonic ENS was examined using affinity-purified antisera against various Goα protein subtypes (including Gsa, Giα, Goα, and Gfα, Quan et al., 1993). Initially, during the slow, circumferential phase of migration (40-50%), no G protein expression was seen in the EP cells, which during this stage formed a coherent packet of cells adjacent to the foregut-midgut boundary (structure labelled ‘EP’ in Fig. 1B; see also Fig. 2A). Goα immunoreactivity could be detected within processes of the recurrent nerve, descending from the more anterior ganglia (Figs 1B, 2E, arrow). Then just prior to the dispersive phase of migration (55-60%), faint levels of Goα immunoreactivity could first be detected in the EP cells that had become aligned with the newly formed muscle bands on the adjacent midgut (Fig. 1B, stippled clusters; Fig. 2E, upper arrowheads). As migration proceeded (58-60%), the intensity of Goα staining gradually increased in the EP cells that had commenced migration. Immunoreactive material could also be traced into the leading neuronal processes that extended along the muscle bands of the midgut (compare TN-1 staining with Goα immunoreactivity; Fig. 2). EP cells moving laterally onto the foregut musculature also began to stain positively for Goα, concurrent with the onset of their migration (Fig. 1B, 58-60%). By ~60% of development, the dispersive phase of migration was largely complete (Copenhaver and Taghert, 1989b); however, the neurons continued to exhibit motile behavior in the form of axonal outgrowth along the gut musculature until 75-80%. During this period, the intensity of Goα immunoreactivity also continued to increase within the EP cell bodies and their processes (Fig. 2H; Fig. 3, arrowheads). Preadsorption of the Goα antiserum with the Go-specific peptide used to generate the antiserum completely blocked all immunoreactive staining (Fig. 3C). Antisera against a number of other Gα subunits, including Gsa (Fig. 3D), produced no detectable staining in either the EP cells or their processes throughout the period of migration.

While Goα-related proteins were first detectable in the EP cells just before the onset of their migration, Goα immunoreactivity could be seen in a number of other tissues at substantially earlier times in development. In the embryonic CNS, Goα immunoreactivity was detectable in the tracts and commissures of the developing ganglia by 35% of development and subsequently could be traced within the axonal processes of all the major nerve roots (Copenhaver and Nichols, unpublished data). Neuropilar staining was also apparent in the frontal ganglion of the foregut (not shown), and as already noted, Goα immunoreactivity could be detected within neuronal processes that descended from the enteric ganglia through the recurrent nerve of the foregut (Figs 1B, 2E). Several non-neural tissues also exhibited positive staining for Goα during embryonic and postembryonic development, including the gonads and prothoracic glands (not shown). Transient staining of the midline epithelium of the midgut was also apparent between 55 and 60% of development, coincident with the final closure of the midgut and overgrowth of the visceral musculature (see arrow at 58% in Fig. 1B; arrow in Fig. 2G). This staining was clearly distinguishable from the immunoreactive material within the migratory EP cells. It should be noted that several other classes of G proteins could also be detected by immunohistochemistry in the developing CNS of Manduca, including Giα, Gsa, and Gfα (Copenhaver and Lagrange, unpublished observations). However, none of these additional antisera resulted in positive staining of the EP cells, supporting our conclusion that these neurons selectively express Goα-related proteins around the time of their migration.

Post-migratory regulation of Goα expression

While the initial appearance of Goα in the EP cells coincided with the onset of their migratory behavior, the levels of Goα immunoreactivity continued to increase in the enteric plexus even after migration was complete (Figs 2H, 3B). When we examined the distribution of Go-related proteins in the postembryonic ENS, however, we found that the pattern of Goα expression had changed significantly. Whereas all of the EP cells initially stained positively for Goα during migration, only certain subsets of the neurons remained immunoreactive in postembryonic stages (Fig. 4A). Specifically, all of the EP cells that had migrated onto the foregut musculature continued to exhibit Goα immunoreactivity (Fig. 4B), although the level of staining in these cells appeared more diffuse and was less clearly associated with the plasma membrane than in embryonic stages. Within the neuronal populations of the
Fig. 1. Onset of Goα expression during cell migration in the ENS. (A) Schematic representation of the mature distribution of neurons (EP cells) in the ENS. Boxed panel shows a dorsal view of the enteric plexus on either side of the foregut-midgut boundary (circled arrow). The enteric plexus is connected with the anterior enteric ganglia of the foregut by the recurrent nerve (RN); branches of the plexus extend onto specific sets of muscle fibers on the foregut and along the eight major longitudinal muscle bands of the midgut (L1-L4 and R1-R4). (B) Camera lucida drawings of the developing enteric plexus at successive stages of embryogenesis (shown in percentage development) to illustrate the onset and enhancement of Goα immunoreactivity during EP cell migration. Each panel shows a dorsal view of the embryonic gut in the vicinity of the foregut-midgut boundary (anterior is to the top). At 50%, the EP cells form a discrete packet of premigratory cells (labelled EP; compare with Fig. 2) that sits adjacent to the foregut-midgut boundary; no detectable Goα immunoreactivity is present in the EP cells, although there is staining in processes descending through the recurrent nerve (RN) from the anterior enteric ganglia (ganglia are not shown). At 55%, faint levels of staining can be seen in the leading groups of EP cells (stippled cell groups) and in their processes that have extended onto the midgut muscle bands (arrowheads). From 55-60%, as the EP cells migrate onto the visceral musculature of both the foregut and midgut, there is a gradual enhancement of staining in all of the migratory neurons and their processes (arrowheads indicate the growing tips of EP cell processes). Transient staining also appears within the epithelium of the midgut at the dorsal midline at ~58% (arrow), during the final phase of midgut closure. Following the completion of migration (65%), the level of staining continues to increase both in the somata of the EP cells and in their processes as they continue to elongate along the gut musculature (arrowheads). Curved arrows depict the general directions of neuronal migration. Scale: 0.05 mm.
midgut, we found robust levels of Go\text{α} immunoreactivity, but only in a subset of the EP cells that had migrated onto the muscle bands (Fig. 4C). When preparations were also labelled with an antiserum that recognized FMRFamide-related peptides in the ENS (Fig. 4D), we found that only the EP cells that exhibited this peptidergic phenotype (the ‘type B’ cells; Copenhaver and Taghert, 1989a) also continued to stain positively for Go\text{α}. Thus the expression of Go\text{α} within the enteric plexus showed an additional phase of regulation once migration was complete, corresponding with the differential expression of mature phenotypes by distinct subsets of the EP cells. These observations suggest that Go may serve an additional function within the ENS that is specific to the peptidergic class of post-migratory neurons.

**Immunoblot analysis of Go\text{α} expression**

To characterize the proteins being recognized by the anti-Go\text{α} antiserum in *Manduca,* we also performed an immunoblot analysis of extracts from a variety of embryonic and postembryonic tissues. As shown in Fig. 5, a single moiety having an approx. $M_t$ of $40\times10^3$ was recognized by the anti-Go\text{α} antiserum when applied to nitrocellulose blots of protein separated in a polyacrylamide gel. The tissue distribution of labelled proteins in these blots corresponded well with the patterns of immunoreactivity that were revealed by the anti-Go\text{α} antiserum in our whole-mount preparations. In postembryonic animals, substantial amounts of the $\sim40\times10^3$ $M_t$ protein were present in extracts of larval CNS (lane B), larval midgut muscle bands (which included the EP cells and their
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Fig. 3. Goα immunoreactivity is eliminated by preadsorption with Goα-specific peptides. (A) TN-1 stained embryo at 65% of development shows the distribution of EP cells on the mid-dorsal muscle bands of the midgut, following the completion of migration. (B) A different embryo of the same age, stained with the anti-Goα antiserum; intense immunoreactivity is present in both the cell bodies (arrows) and in their processes that extend posteriorly along the midgut muscle bands (arrowheads). (C) Goα immunoreactivity is completely blocked when the antibody is pre-incubated with the Goα-specific peptide used to generate the Goα antiserum. Unstained cell bodies (arrows) and the approximate positions of their processes (arrowheads) can be faintly seen using Nomarski optics. (D) An antiserum against the α-subunit of Gs produces no detectable staining in either the EP cells or their processes. Scale: 50 μm.

processes; lane C), and the enteric plexus regions of hatching midguts (lane F). In contrast, no proteins were labelled in extracts from larval fat body (lane A) or from interband regions of the midgut (regions that contains visceral muscle but were devoid of EP cells; lanes D, E), nor was any positive signal seen in extracts from skeletal muscle or malpighian tubules (not shown). In pooled tissues from embryos dissected at 75% of development, a protein band of similar molecular mass was detected in extracts from both embryonic midgut (containing the postmigratory EP cells; lane G) and in the embryonic body (which included the developing CNS; lane H). A second labelled band of ~30 × 10^3 M_r was also seen in the latter sample. The apparent size of the major protein band closely matched the molecular mass of the Goα subunit in extracts from Drosophila brain (lane I; see also Thambi et al., 1989). As with our immunohistochemical staining, all positive signals were completely abolished when the anti-Goα antiserum was preadsorbed with the synthetic Goα peptide, prior to the application of the antiserum to the blots.

Effects of AlF_4− and GTPγ-S on EP cell migration

The appearance of Goα-like proteins in the EP cells coincident with the onset of their migration suggested that G proteins might play a role in regulating one or more aspects of the migratory process. To investigate this possibility, we exposed the EP cells in semi-intact embryos to varying concentrations of AlF_4−, a compound that has been shown to activate all heterotrimeric G proteins in an unregulated manner (Sternweiss and Gilman, 1982; Anderson et al., 1991) but does not affect the small-molecular mass family of ras-related proteins (e.g. Donaldson et al., 1991). Embryos were opened in culture starting at about 52% of development (±0.5%), at which time Goα immunoreactivity could be faintly detected in the EP cells but the migratory dispersal of the neurons had not yet commenced (Fig. 6A). When control embryos were permitted to develop in culture for 6-8 hours and then fixed and stained with TN-1, we observed that all aspects of EP cell migration and process outgrowth proceeded normally (Fig. 6B). In contrast, when identically staged embryos were cultured in the presence of AlF_4−, we subsequently found a dramatic inhibition of both EP cell migration and process outgrowth (Fig. 6C-E), although the overall appearance of the neurons and their expression of the surface epitope recognized by TN-1 remained relatively normal. The examples shown in Fig. 6 were chosen from six separate experiments (employing a total of 53 experimental preparations) to illustrate the range of effects that we observed following exposure to AlF_4−. In most of these preparations, however, we saw little or no evidence of migratory dispersal onto the musculature of the foregut and midgut.

We also examined the effects of AlF_4− exposure at several different stages around the onset of EP cell migration (Table 1). When cultured embryos were exposed to AlF_4− for various periods between 45 and 50% of development, we observed only minor effects on the timing and number of EP cells that subsequently migrated onto the midgut, although AlF_4− did impede the normal closure of the gut epithelium. In contrast, treatment with AlF_4− between 50 and 55% (just prior to migration onset) substantially inhibited both the migration and outgrowth of the EP cells, as also shown in Fig. 6. At still later stages, when the EP cells were actively dispersing along the muscle bands (55-60%), treatments with AlF_4− continued to have an inhibitory effect on both cell migration and process outgrowth when compared with matched controls. Thus the onset of sensitivity to AlF_4− treatment coincided reasonably well with the appearance of Goα immunoreactivity in the EP cells (first detectable at ~54% of development; Fig. 2) and persisted throughout the period of their active migration. Similar treatments with AlF_4− were also found to inhibit process outgrowth from the CNS of Manduca (Nichols and Copenhaver, unpublished observations), coincident with the expression of Goα immunoreactivity in the growing axons.

To take into account non-specific effects that might result
from a continuous exposure to aluminium salts, we performed an additional set of experiments in which alpha toxin was used to enhance the permeability of the EP cells to an abbreviated pulse of AlF$_4^-$ when embryos at 52% of development were exposed to alpha toxin alone for 20-30 minutes and then allowed to develop for an additional 8 hours in normal medium, no significant changes were detected in either the number of EP cells that migrated or in the extent of neuronal migration and process outgrowth (Fig. 7, hatched bars). Similarly, when embryos were exposed first to AlF$_4^-$, rinsed, and then exposed to alpha toxin over the course of 30 minutes, no diminution in the rate or extent of EP cell motility were subsequently observed (chequered bars). In contrast, both cell migration and process outgrowth were subsequently reduced when embryos were exposed to a combination of alpha toxin and AlF$_4^-$ for 20-30 minutes, followed by extensive rinsing with normal medium (black bars). Exposure to AlF$_4^-$ by itself during this same period had only modest effects on the number or extent of migratory neurons (stippled bars); in some of these preparations, however, there was a substantial inhibition of process outgrowth, resulting in the large standard deviation shown in histogram III-D. As with longer exposures to AlF$_4^-$ alone (Fig. 6), the presence of alpha toxin in combination with AlF$_4^-$ produced no obvious deleterious effects on the TN-1 staining in the enteric plexus or on the general morphology of the EP cells, despite their diminished motility.

These experiments showed that a brief exposure of the EP cells to AlF$_4^-$, a compound that has been shown to stimulate the activation of G proteins in other systems, caused a dramatic inhibition of neuronal motility in the developing ENS.

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**Fig. 4.** Postembryonic expression of Go$_{11}$ in the enteric plexus is restricted to specific subsets of the differentiated EP cells. (A) Camera lucida image of the enteric plexus in a third instar larva, stained with the anti-Go$_{11}$ antiserum. All of the EP cells that migrated onto the foregut retain detectable levels Go$_{11}$-related protein, although the distribution of immunoreactive material in these neurons appears more diffuse than during their migratory stage of embryonic development. (B) A photomicrograph of EP cells (arrows) on the larval foregut, stained with the anti-Go$_{11}$ antiserum and visualized by immunofluorescence with a fluorescein-conjugated marker. In contrast, only a subset of the EP cells that have migrated onto the midgut still contain detectable levels of Go$_{11}$-related protein, so that immunopositive and immunonegative neurons are intermingled along the midgut muscle bands (filled and clear cells in panel A). (C) One cluster of EP cells on a midgut muscle band, stained with the anti-Go$_{11}$ antiserum and a fluorescein-conjugated secondary antiserum; a subset of the neurons and their processes are clearly stained (solid arrows), with immunoreactive material primarily in the vicinity of the plasma membrane. The other EP cells show no evidence of Go$_{11}$ immunoreactivity (open arrows). (D) The same preparation stained with a monoclonal antibody that recognizes FMRFamide-related peptides in the ENS, visualized with a rhodamine-conjugated secondary antibody. Only those neurons that exhibit the peptidergic phenotype still express detectable levels of Go$_{11}$ immunoreactivity (compare with C).
G proteins in neuronal migration

Table 1. Effects of AlF₄⁻ treatment on EP cell motility at various stages of development

<table>
<thead>
<tr>
<th>Developmental stage of AlF₄ treatment</th>
<th>% Inhibition of cell migration (vs. controls)</th>
<th>% Inhibition of process outgrowth (vs. controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45-50% (pre-migration)</td>
<td>5</td>
<td>(n.m.)</td>
</tr>
<tr>
<td>50-55% (migration onset)</td>
<td>85</td>
<td>83</td>
</tr>
<tr>
<td>55-60% (mid-migration)</td>
<td>97</td>
<td>90</td>
</tr>
</tbody>
</table>

Exposure of cultured embryos to AlF₄⁻ at times between 45 and 50% of development (prior to the appearance of Goₐ immunoreactivity in the EP cells) caused only minor effects on the subsequent migration of the EP cells when compared to controls; process outgrowth in these preparations was not measured (n.m.). Treatments applied between 50 and 55% of development (around the onset of Goₐ expression in the EP cells) resulted in a substantial inhibition in both neuronal migration and process outgrowth (compare with Fig. 7). Treatments applied at still later times during migration (between 55 and 60%) continued to have a strong inhibitory effect on both aspects of EP cell motility. Data represent the pooled averages from several different experiments involving a minimum of five animals per experiment.

However, while AlF₄⁻ does not activate the ras-related family of GTP-binding proteins (Donaldson et al., 1991), it has been shown to stimulate a number of other metabolic enzymes that are unrelated to the heterotrimeric G proteins (e.g. Lange et al., 1986; Robinson et al., 1986). As a means of corroborating our results with AlF₄⁻, we also initiated a series of experiments using intracellular pressure injections of individual EP cells with other compounds that affect G protein activity. When individual EP cells were injected, prior to the onset of migration, with a control solution containing the fluorescent marker LRD (Fig. 8A), we subsequently observed that most of the injected cells continued to exhibit both normal migration and process outgrowth (Fig. 8B). In contrast, when individual cells were injected with GTPyS, a non-hydrolyzable form of GTP that has been shown to activate all heterotrimeric G proteins (Stryer and Bourne, 1986; Gilman, 1987), we observed a substantial reduction in the extent of both migration and outgrowth in approximately 50% of the injected cells. These results, though preliminary, support our experiments with AlF₄⁻ and suggest the extent of EP cell migration may be regulated via the activation of one or more G protein-mediated events in the course of neuronal development.

DISCUSSION

Developmental expression of G proteins in the ENS

These results show that a protein related to the Go class of heterotrimeric G proteins is selectively expressed by a defined population of migratory neurons, the EP cells, and that the appearance of this protein coincides with their transition from a premigratory to a migratory stage of development. An important consideration in evaluating these results is the sensitivity of our methods for detecting G proteins in the developing ENS. Several lines of evidence support our conclusion that the EP cells express only one type of G protein, Go, coincident with the onset of their migration. As noted above, we screened embryos with a number of antisera against other G protein subtypes (including Gs, Gi, Gt and Gf; Quan et al., 1993), all of which produced distinctive patterns of immunoreactive staining in the CNS and other regions of the embryo (Copenhaver and Lagrange, unpublished observations). We also could detect Go-related material in the CNS and several nerve branches (including the recurrent nerve of the foregut; Fig. 2) at significantly younger ages than in the EP cells, indicating that our failure to detect G proteins in the premigratory EP cells was not simply due to stage-specific levels of expression in the embryo as a whole. G proteins have also been revealed by these same methods in the developing fly embryo (Wolfgang et al., 1991), in which a number of G protein subtypes (including Go) have been detected from the earliest stages of embryonic development; the presence of G proteins in the ENS of Drosophila has not been examined, however.

With respect to the identity of the molecules that were recognized by the anti-Goₐ antiserum, several lines of evidence support our conclusion that the EP cells express an authentic form of Goₐ during their migration. As noted in Fig. 3, Goₐ immunoreactivity in the EP cells was completely suppressed when the antiserum was preadsorbed with the Goₐ-specific peptide that was initially used to generate the antiserum (Goldsmith et al., 1987). In addition, Wolfgang et al. (1990) have shown that immunoreactive staining with this antiserum in Drosophila is not inhibited when the antiserum is preadsorbed with peptides specific to other G proteins. Thus in the insect nervous system, the anti-Goₐ antiserum appears to recognize members of the Go class of proteins selectively. Further support for this conclusion was found in our immunoblot analysis of protein extracts, in which this same antiserum recognized a single band of appropriate molecular...
mass (~40 × 10^3) in a tissue-specific manner, corresponding to the distribution of immunoreactive staining in whole-mount embryonic preparations. A single protein band of similar molecular mass has been found in tissue extracts from Drosophila, and this protein has been shown to correspond to authentic Go (Thambi et al., 1989; Fig. 5, lane I). The appearance of an additional band of lower molecular mass in extracts of embryonic body (Fig. 5, lane H) might be due to a degradation product of authentic Goα or might reflect cross-reactivity of the antiserum with another protein; however, this additional band was never seen in extracts that included the EP cells. Again, all positive signals in the immunoblots were completely blocked by preadsorption of this antiserum with Go-specific peptides.

While we believe that the immunohistochemical procedures used in this paper provide a sensitive means of detecting Go-related proteins in the ENS, these results will require confirmation with an analysis of Goα gene expression in this system. We have recently characterized a clone from a Manduca cDNA library that contains all the major features of Goα in other systems (Horgan, Lagrange and Copenhaver, unpublished data), including the conserved peptide sequence (ANNLRGCGLY) that was used to generate the anti-Goα antiserum. Using probes derived from this clone for in situ hybridization histochemistry, we have now been able to detect the presence of Goα-specific mRNAs in the EP cells just prior to their migration. This approach can now be used to examine the developmental expression of Goα during neuronal migration in more detail.

**Role of G proteins in EP cell migration**

The initial appearance of Goα-related proteins in the EP cells just prior to the onset of migration suggests a number of possible functions for Go in the migratory process. One obvious possibility is that the expression of functional G proteins in the neurons is required for the initiation of migratory behavior. For example, Go might be coupled to a membrane-bound receptor that recognizes extracellular cues for migration, and in turn might cause a stimulation of the intracellular machinery that underlies cellular motility. Alternatively, the initial expression of G proteins might simply be coincident with the onset of migration, in advance of some later aspect of migratory behavior in which G protein-mediated events are required. As previously described, the migration of the EP cells is tightly regulated, both in terms of directionality and duration: EP cell migration lasts from 55 to 60% of development and then abruptly stops (Copenhaver and Taghert, 1989b), and the EP cells will migrate on the eight muscle bands of the midgut but not the adjacent visceral musculature (Copenhaver and Taghert, 1988). Lastly, Go might serve no significant function at all during migration, being required instead for some later aspect of EP cell differentiation and appearing at the time of migration by coincidence alone.

**Fig. 6.** Effects of AlF_4^- treatment on EP cell migration in embryonic culture. (A) Staged embryo (52% of development) that was fixed and stained with TN-1 to illustrate the position of the EP cells just prior to AlF_4^- treatment; at this stage, the neurons had not begun the dispersive phase of their migration (foregut-midgut boundary is indicated by the horizontal black line in each panel). (B) Embryo that was placed in culture at 52% (same age as panel A) and allowed to develop in normal medium for 8 hours before fixation and staining with TN-1. Both cell migration and process outgrowth proceeded normally under these conditions. (C-E) Embryos that were placed in culture at 52% in the presence of 20-40 µg/ml AlF_4^- and allowed to develop for 8 hours. Arrowheads indicate the most dispersed groups of EP cells in these preparations. Preparations in C-E were chosen to illustrate the range of migration that was observed under these conditions. Scale: 50 µm.
As an initial means of distinguishing among these possibilities, we have used AlF_4- to stimulate all G protein activity in the EP cells just prior to migration onset. As shown in Figs 6 and 7, we found that stimulation of G protein activity caused a marked inhibition of neuronal motility. These results support a role for Go in regulating the extent or perhaps directionality of EP cell migration, as opposed to providing the initial trigger for migration (in which case stimulation of Go should cause precocious or exuberant migration). For example, the motile behavior of the EP cells might be limited in part by a variety of membrane- or matrix-associated cues associated with their migratory environment, as has been demonstrated for migratory cells in a number of other preparations (Heaysman et al., 1987; Bronner-Fraser, 1990; Baird et al., 1992; Le Douarin and Dupin, 1993). By this scenario, detection of these inhibitory cues would involve Go-coupled membrane receptors in the EP cells, which when stimulated would cause a termination of migratory behavior and a transition to a subsequent phase of differentiation (including the expression of mature neuronal phenotypes; Copenhaver and Taghert, 1989a; 1989b). Moreover, we found that treatment with AlF_4- caused an inhibition of neuronal motility throughout the period of EP cell migration (Table 1), whereas treatments applied before the onset of GoQ expression did not cause a significant disruption in migratory behavior.

Our results support the hypothesis that a Go-mediated pathway plays an important role in regulating the motile behavior of the EP cells: both cell migration and process outgrowth were markedly inhibited in the presence of AlF_4-, a compound that is known to induce the activation of all heterotrimeric G proteins (Sternweiss and Gilman, 1982; Anderson et al., 1991), but not members of the ras-related family of GTP-binding proteins (Donaldson et al., 1991; Ktistakis et al., 1992). Several important caveats must be included in the interpretation of these experiments, however. While we have failed to detect other G proteins in the EP cells during migration, the possibility that unidentified members of this family are present at low levels or are unrecognized by our antisera cannot be excluded. It is also conceivable that the effects of AlF_4- were indirect, resulting from a disruption of the migratory pathways of the enteric plexus. In most preparations, however, all of the morphological features of the gut (including the differentiated midgut bands of visceral musculature) could still be distinguished, indicating that the normal pathway structures were nominally present. Similarly, it might be argued that the effects of AlF_4- were simply due to the general toxicity of aluminum salts or to an increase in the osmolarity of the culture medium. The fact that the pore-forming compound alpha toxin potentiated the effects of AlF_4-, under conditions in which neither AlF_4- nor alpha toxin alone inhibited migration argues against this possibility. Moreover, increasing the osmolarity of our culture medium with mannitol by as much as 60 mosmol has previously been found to have no major effects on either EP cell migration or process outgrowth (Copenhaver and Lagrange, unpublished observations).

Beyond the limitation that AlF_4- acts as a non-selective stimulating agent for all G proteins, the use of AlF_4- is further complicated by its reported effects on a number of other metabolically important molecules, including glucose-6-phosphatase (Lange et al., 1986), mitogen-activated protein kinases (Anderson et al., 1991), and Na^+-K^+-dependent ATPase (Robinson et al., 1986). A number of more selective reagents have been described that can be used to manipulate G protein activity, including non-hydrolyzable forms of GDP and GTP (Stryer and Bourne, 1986; Gilman, 1987). Our preliminary results using intracellular pressure injections of GTPγS (Fig. 8) provides support for the data obtained with AlF_4-, in that the introduction of a compound that should stimulate all heterotrimeric G proteins also caused an inhibition of migration. Unlike AlF_4-, GTPγS can also stimulate members of the ras-related family of guanyl nucleotide-binding proteins (Donaldson et al., 1991; Ktistakis et al., 1992), so these results must similarly be viewed with caution. Several toxins that perturb certain subsets of heterotrimeric G proteins have also been described, including pertussis toxin (e.g. Tamura et al.,

![Fig. 7. Effects of a brief exposure to AlF_4- on EP cell migration in the presence of the pore-forming compound, alpha toxin. Embryos were placed in culture at 52% of development (as in Fig. 6), allowed to develop for 6 hours at 28°C, and then stained with TN-1. Camera lucida images of these preparations were then used to calculate (I) the number of migratory EP cells, (II) the greatest distance travelled by an EP cell, and (III) the greatest distance of process outgrowth beyond the EP cells on each of the dorsal muscle bands (L1-L2 and R1-R2; see Fig. 1A). Results were then normalized for each band with respect to values taken from control animals; the average values and standard deviations are given for each treatment group above the bars (numbers indicate the number of preparations used for each treatment). Statistical values were calculated with respect to a smaller number of matched control animals in each experiment, using a one-tailed Student’s t-test. (A; open bars) Control values; error bars were derived from pooled controls from several different experiments to illustrate the normal variation in migration and outgrowth. (B; hatched bars) A brief (20-30 minute) exposure to 0.02-0.01 mg/ml alpha toxin alone followed by extensive rinsing produced no significant changes in the number of EP cells that migrated or in the distance that they migrated along the muscle bands. (C; chequered bars) Sequential treatments with 25 μg/ml AlF_4- alone (for 10 minutes), rinsing in normal medium (for 10 minutes) and then alpha toxin alone (for 10 minutes) similarly caused no diminution in either cell migration or process outgrowth. (D; stippled bars) Exposure to 25 μg/ml AlF_4- alone for (20-30 minutes) produced no significant changes in the number of EP cells that migrated or in the distance that they migrated along the muscle bands, although in some of these preparations there was a substantial inhibition in process outgrowth (note the large standard deviation for III-C). (E; black bars) Exposure of the EP cells to AlF_4- in the presence of alpha toxin caused a significant inhibition of all three aspects of motility: *P<0.05 for the number of migrating EP cells (I) and the distance of process outgrowth (III); **P<0.01 for the distance of the greatest distance of EP cell migration (II).]
G proteins and the regulation of directed cellular motility

Support for the regulation of EP cell migration by Go can be drawn from work on another form of neuronal motility, the outgrowth of processes during axogenesis. Several different classes of G proteins have been localized to regions of active outgrowth and synaptogenesis in the CNS of both vertebrates and invertebrates (Chang et al., 1988; Pituello et al., 1991; Wolfgang et al., 1991; Asano et al., 1992), and high concentrations of both Gi and Go have been found in association with growth cone membranes (Edmond et al., 1990; Strittmatter et al., 1990; Garibay et al., 1991). Strittmatter et al. (1990) have also shown that Go subunits can be activated by another growth cone protein, GAP-43, whose expression is correlated with axonal extension and may stimulate filopodial extension (Goslin et al., 1988; Zuber et al., 1989); the functional consequences of this interaction are as yet unknown.

Other studies have indicated that G proteins may serve a variety of functions in motile growth cones, depending on the environmental context in which the cells are growing. In PC-12 cells that have been treated with nerve growth factor, process outgrowth in response to several different cell adhesion molecules is mediated in part by pertussis-sensitive G proteins (Doherty et al., 1991, 1992), which appear to stimulate growth cone motility via the activation of one or more calcium channels (Schuch et al., 1989; Doherty et al., 1991). In other preparations, pertussis-sensitive G proteins have been implicated in the inhibition of calcium currents in growth cones (Man-Son-Hing and Haydon, 1992). As demonstrated in a number of recent studies, the regulation of intracellular calcium may in turn affect growth cone motility in a variety of ways, depending on the developmental context and the particular cell type involved (e.g. Goldberg, 1988; Mattson et al., 1988; Silver et al., 1989; Rehder and Kater, 1992).

Recently, pertussis-sensitive G proteins have been strongly implicated in the inhibition of responses of growth cones to several different collapsing factors from embryonic and postembryonic brain tissue (Igarashi et al., 1993). These results suggest that G proteins may play a critical signalling function during the guidance of axons through the substrates of the developing brain and peripheral nervous system. While the mechanisms by which G proteins regulate growth cone motility are unknown, G proteins have been shown to affect the polymerization of several major cytoskeletal proteins, either directly or indirectly (Berlot et al., 1987; Wang et al., 1990; Cooper, 1991), which ultimately must underlie the motile behavior of neurons and their growing processes (Singer and Kupfer, 1986; Smith, 1988; Cooper, 1991).

G proteins have been implicated in the directed migration of non-neural cell types, as well. Among the most extensively examined, the migratory behavior of both polymorphonuclear leukocytes and the slime mould Dictyostelium in response to diffusible chemotactic factors have been shown to require the activation of membrane-associated, G protein-coupled receptors (Devreotes and Zigmond, 1988; Caterina and Devreotes, 1991). These in turn may stimulate a variety of intracellular messenger systems (Firtel et al., 1989; Hall et al., 1989; Newell, 1990) and result in specific changes in locomotory behavior. The mechanisms by which these intracellular signalling pathways induce chemotaxis are still enigmatic; however, and in the case of neuronal migration, the role of G proteins in governing particular aspects of cellular motility remains largely unexplored.

Differential regulation of Go expression

A final point that deserves consideration is the differential expression of Go in the EP cells once their mature neuronal phenotypes have been established. As already noted, all of the EP cells began to show positive immunoreactivity for Go in the midgut around the time of their migration, indicating that the initial expression of the Go gene was uniformly regulated within this cell population. In contrast, by the time the EP cells completed their terminal differentiation, only certain subsets of the neurons still contained detectable levels of Go-related proteins (Fig. 4). Among the EP cells that had migrated onto the midgut, only those neurons that had acquired a specific peptidergic phenotype (related to the FMRFamide class of neuropeptides) still stained positively with the Go-specific antiserum. As previously demonstrated, the expression of this peptidergic phenotype occurs in a position-specific manner and is delayed until after migration is complete: FMRFamide immunoreactivity appears only in the ‘type B’ subset of EP.
cells that occupy the migdut domains of the enteric plexus, and the timing of expression of this phenotype is regulated in part by interactions with the post-migratory environment of the migdut (Copenhaver and Taghert, 1988; and unpublished observations). Thus epigenetic interactions may similarly contribute to the differential regulation of the GoX gene within the EP cells during the establishment of mature neuronal phenotypes in the ENS. While Go-like proteins have previously been associated with regions of active neuronal migration or process outgrowth (as noted above), the subsequent expression of G proteins with respect to mature neuronal phenotypes has not been characterized. The accessibility of the ENS throughout the period of neuronal migration and differentiation should now permit us to explore these issues in the context of a normally developing embryo.

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