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Recognition of Specific Targets by Cultured Dorsal Root Ganglion Neurons

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We have assessed the effects of different target cell populations on axonally transported proteins by the use of compartmental cell culture systems that separate the soma from the growing axons of rat sensory neurons. The labeling of 3 rapidly transported proteins diminishes when the growing axon contacts spinal cord cells (which are normal *in vivo* targets), and remains unaffected by contact with fibroblasts or heart cells. Medium conditioned by spinal cord cells does not exert this effect. Thus, specific classes of cells may be distinguished as target tissue by sensory neurons *in vitro*. Such recognition is accompanied by specific molecular changes in axonally transported proteins.

The transition from nerve growth cone to nerve terminal and the consequent formation of specific connections in the developing vertebrate nervous system rely especially on interactions of the neurons with their target tissues (Cowan et al., 1984; Easter et al., 1985). Thus, trophic factors from appropriate postsynaptic cells may enhance neurite outgrowth (Varon and Adler, 1981; Barde et al., 1983; Berg, 1984), and neuronal cytostructure may reflect contact with target tissues (Denis-Donini et al., 1983). This implies that the growing neuron possesses molecular machinery that recognizes specific microenvironments. Additional cellular transduction mechanisms must direct either continued growth cone movement or cause cessation of movement and the generation of a stable nerve terminal structure. In theory, the molecules integral to these activities can be identified as those whose expression is selectively regulated by different cellular environments. This work represents an attempt to identify such molecules in rat dorsal root ganglion neurons.

In previous work (Sonderegger et al., 1983) we noted that such regulated molecules are likely to constitute only a small proportion of the complement of cellular proteins. Thus, total cellular proteins, analyzed by 2-dimensional gel electrophoresis, reveal no differences when neurons are compared prior to and after synapse formation. However, a few of the subset of cellular proteins that are axonally transported are regulated by contact with target cells. Similar regulation of axonally transported proteins has been suggested by work using the *Aplysia* R_2 neuron (Ambron et al., 1985a, b). In view of the pronounced specificity of cell–cell interaction in the nervous system (e.g., Easter et al., 1985), our initial hope was that some of these "target-regulated" proteins might evidence selective regulation by different targets. Pursuit of the originally noted proteins revealed no such specificity, although computer analysis of the 2-dimensional electrophoretic patterns suggested that some minor constituents were regulated differently by different targets (Sonderegger et al., 1985). One subset of neuronal proteins that seems an especially attractive candidate for modulation by cell–cell interactions is that which is rapidly transported, since it includes proteins destined for distal membrane insertion (Tyell et al., 1981). The compartmentalized culture system that we have used in previous investigations was redesigned to facilitate study of these proteins.

Here we report the nature of specific target cell recognition by rat sensory neurons, as evidenced by the regulation of 3 rapidly transported axonal proteins.

Materials and Methods

Cell culture. Dorsal root ganglia (DRG) were dissected aseptically from 19 to 20 d (E19–20) embryonic rats and dissociated by incubation in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS) containing 0.1% trypsin, 0.1% collagenase, and 0.01% DNAse (all wt/vol) for 30 min at 37°C. After trituration with a flame-polished pasteur pipette, approximately 250 μl of the cell suspension was plated within a rectangular Teflon chamber 4 mm wide × 40 mm long × 6 mm deep. This chamber was attached to a 60 mm culture dish with Dow Corning high-vacuum grease. (A schematic diagram of this culture system is presented in Fig. 1.) The culture dish had previously been coated with collagen (Vitrogen) and scratched with a linear array of needles in order to direct axon growth, as previously described (Campenot, 1977; Sonderegger et al., 1983). The culture chambers in the present experiments held approximately 5–8-fold more cells than chambers used previously, an improvement that allowed assessment of the rapidly transported proteins.

The cultures were maintained in F14 medium (Vogel et al., 1972), with the addition of 5% fetal bovine serum and 50 ng/ml nerve growth factor purified by the method of Mobley et al. (1976). Cytosine arabinoside (Ara-C; 10^{-5} M) was added between days 4 and 7 after plating in order to eliminate non-neuronal cells. When the effects of other cells on DRG axonal proteins were to be assessed, these other cells were plated in the culture dish outside of the chamber. For each experimental comparison (e.g., control DRG axons versus those contacting target tissue), one plate was used as control and one as test, and these were plated on the same day from the same mixture of cells. Each specific comparison was repeated 4–6 times, as indicated in the figure legends.

Muscle cells were dissected from E18 rat hindlimb and dissociated and plated as described previously (Fishman and Nelson, 1981). Spinal cords were obtained from E15 rats and dissociated in Ca^{2+}- and Mg^{2+}-free HBSS containing 0.1% trypsin and 0.01% DNAse (wt/vol) for 20 min at 37°C. Because spinal cord cells survive poorly in the F14 medium used for DRG, the experiments using spinal cord cells were performed in medium consisting of F14 with 10% horse serum, 10% fetal calf serum, nerve growth factor, and 6 gm/liter additional glucose (Ransom

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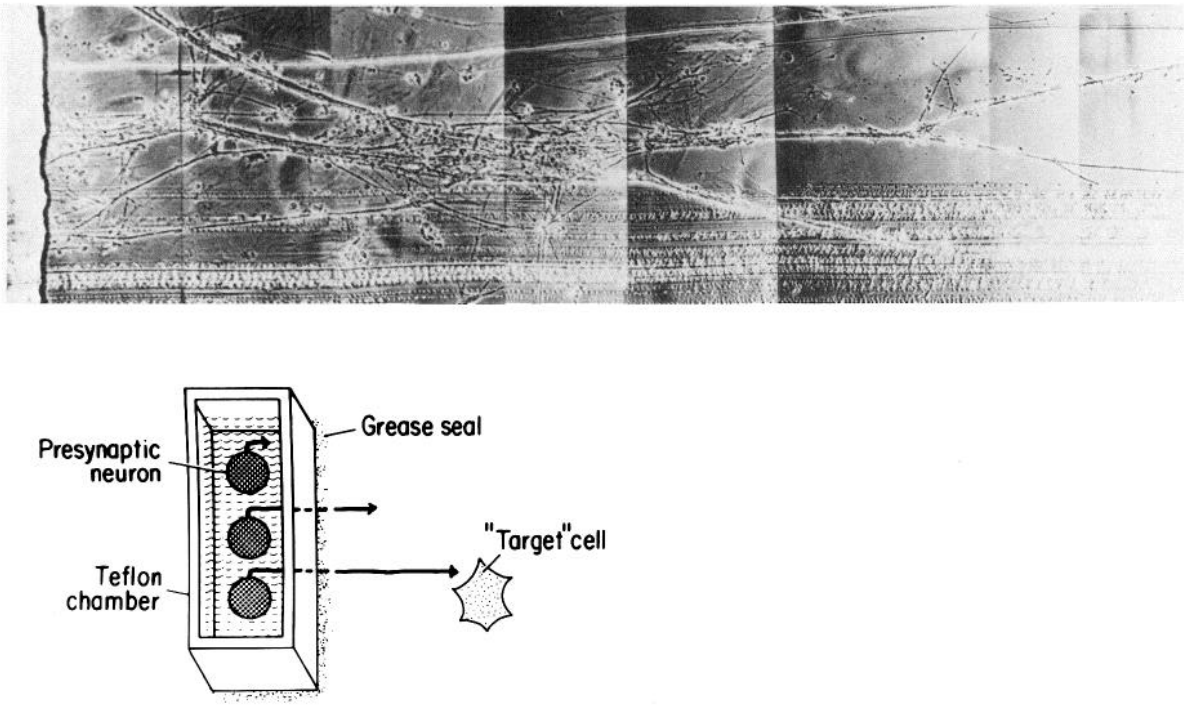

Figure 1. Compartmental culture system used in these experiments. *Above,* Phase-contrast photomicrograph of growing sensory neuronal axons in outer area of culture dish. *Below,* Schematic diagram of neurons cultured in this system.

et al., 1977). Control DRG cultures for these experiments were maintained in the identical medium. Skin fibroblasts were obtained from E19–20 rats by the method of Grumet et al. (1983). The day after plating target cells, the medium was replaced and the cultures maintained for 4 d prior to labeling. The effects of soluble factors were assessed by plating dissociated E15 spinal cord cells within circular Teflon chambers that had been attached to the culture dish in a manner that prevented axonal egress but that allowed sharing of culture medium between spinal cord and DRG cells.

Metabolic labeling. Thirty microliters of methionine-free F14 with 50 ng/ml nerve growth factor containing 200–500 μCi ^{35}S-methionine (spec. act., 1000 Ci/mmol) was added to the chamber. Cultures were subsequently incubated at 37°C for varying periods of time as described. The labeled axons and growth cones in the outer compartment were harvested by solubilization with 2% SDS–5% mercaptoethanol. In all cases, leakage between the compartments was first assessed by measuring radioactivity inside and outside the chamber, and only those compartments retaining a ratio of inside to outside radioactivity greater than 250 were used, in order to avoid labeling of cells outside of the chamber.

Two-dimensional gel electrophoresis. Samples were heated to 100°C for 2 min, lyophilized, and resuspended in 2 vol of lysis buffer (O'Farrell, 1975) containing 10% (vol/vol) NP-40. Ten microliters of the samples were reserved for trichloracetic acid (TCA) precipitation in order to assess incorporation of radioactivity into protein. Axonal proteins were resolved by 2-dimensional electrophoresis, with a pH gradient of 4.3–8.0 in the first dimension and an 8–18% acrylamide gradient gel in the second. Approximately 50,000–100,000 TCA-precipitable cpm were loaded onto each gel. The exposure times were adjusted to equalize the product of total radioactivity loaded on the gel times the length of exposure, expressed as cpm-d, for each pair of gels to be compared (Skene and Willard, 1981a; Jacobson et al., 1986). Gels were treated with Enlightening (New England Nuclear), dried, and exposed to Kodak X-OMAT AR x-ray film at −70°C.

The films were quantitated by scanning with a Helena Lab densitometer, using a beam width wide enough to include each spot, and then the intensity of each spot was normalized to the total of the densities of all spots on the film. Neurons from each dissection were divided between plates destined to have targets added and control plates. Usually one litter provided enough cells for about 4 plates. Thus, each test plate had a matching control plated the same day and cultured under identical conditions, but in the absence of target cells. That is why each autoradiographic figure in this paper is shown with its own control: this was done to allow for biological variation due to slight differences in the age of the animals, dissociation conditions, or lots of serum. The mean (normalized) density for each spot, for each target type (±SEM), was compared to the mean (normalized) density for the same spot on the autoradiograms of the matched control plates. Statistical significance was assessed by Student's *t* test. The films were not preflashed, so that linearity of the autoradiographic response was predicted to be imperfect, especially at low levels of exposure. Therefore, only the most heavily labeled proteins of rapid transport were evaluated. The changes noted were robust and easily visible upon inspection of the autoradiograms.

For Western blot analysis, axonal proteins were resolved by 2-dimensional electrophoresis and subsequently transferred electrophoretically to nitrocellulose (Towbin et al., 1979) in a BioRad Trans-Blot apparatus. The blot was reacted with anti-growth-associated protein (GAP)-43 antiserum at a dilution of 1:250 for 12 hr, and bound antibody visualized with horseradish peroxidase-conjugated goat anti-rabbit antiserum (Miles-Yeda).

Results

Embryonic DRG neurons plated within the chamber extend axons under the barrier to the outside compartment within 5–8 d. The chambers (Fig. 1) are designed to allow egress of the axons, but not mixing of medium between somal and axonal compartments. Cultures are labeled metabolically by addition of ^{35}S-methionine to the center chamber, and axonal proteins subsequently analyzed by 2-dimensional gel electrophoresis. Leakage of radioactivity to the outside chamber is minimal. Thus, levels of radioactivity in this compartment are 300–500-fold less than in the center, so that any contaminating non-neuronal cells or plated target cells are unlabeled, or have only their actin and tubulin faintly labeled.

Axonal proteins constitute a subset of total cellular proteins (Fig. 2) which, as *in vivo,* are transported at different rates. Pulse-chase experiments reveal that the more slowly transported components include actin and tubulin, the cytoskeletal components

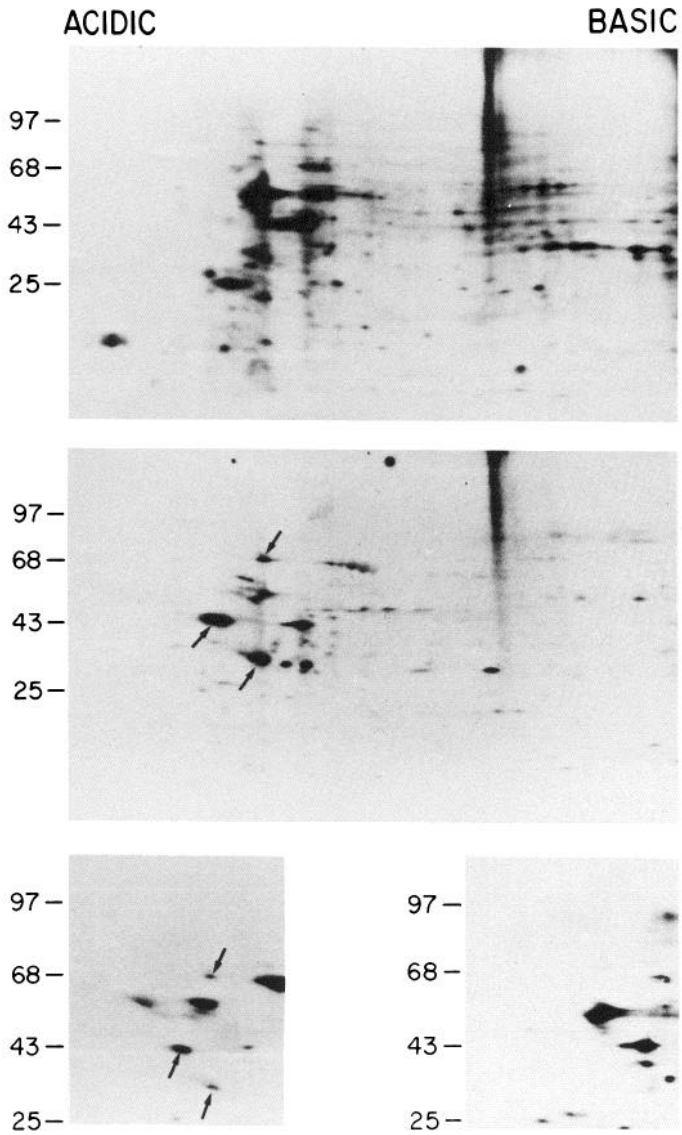

Figure 2. *Top,* Total cellular proteins after 16 hr of continuous exposure of the cell somas to ^{35}S-methionine. *Middle,* Axonal proteins after 16 hr of continuous exposure of the cell somas to ^{35}S-methionine. Note that many of the proteins prominent among the axonal proteins are not abundant, or in most cases even visible, among total cellular proteins. The proteins marked with *arrows* are discussed in the text. *Bottom,* Acidic portion of the gel 2 hr after labeling (*left*) and after a pulse of ^{35}S-methionine followed by a 16 hr cold chase (*right*), demonstrating that the 3 proteins described as regulated in the text (*arrows*) are rapidly transported, and hence visible after 2 hr. After the chase, when slowly transported proteins are evident, the rapidly transported proteins are no longer visible.

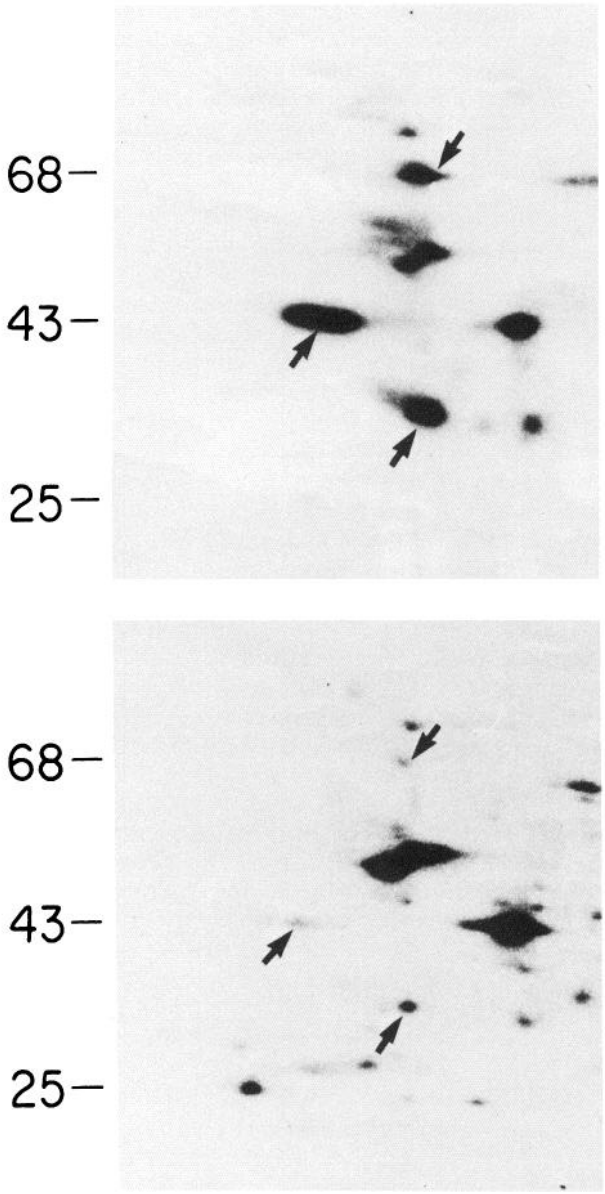

Figure 3. Effect of spinal cord cells plated as target on rapidly transported DRG proteins. DRG neurons were either cultured alone in the compartmental culture system (*upper panel*) or with dissociated spinal cord cells plated in the outer area of the dish (*lower panel*) for 4 d prior to labeling. *Arrows* indicate those proteins that reproducibly exhibit diminished labeling after axonal contact with spinal cord cells. These results were obtained in 5 separate experiments. Actin (about 42 kDa) and α- and β-tubulin (about 55 kDa), by contrast, do not diminish. *Numbers* to the left of each panel indicate molecular weight (in kDa).

demonstrated to be delivered by slow axonal transport *in vivo* (Hoffman and Lasek, 1976). The first appearance of the cytoskeletal proteins about 8–12 hr after the addition of ^{35}S-methionine suggests that the rate of slow transport is comparable to the *in vivo* rate of 2–3 mm/d (Black and Lasek, 1980), given a barrier-plus-seal thickness of about 1–2 mm. Pulse-chase experiments suggest that other proteins are transported at a rate more than 10 times that of the slowly transported proteins (Fig. 2), and these will be referred to as "rapidly transported."

Target cells regulate rapidly transported proteins

Although rapidly transported proteins can be visualized within 2 hr of labeling (Fig. 2), their level of incorporation of ^{35}S-methionine increases with longer incubations, which are consequently used to facilitate the experiments. After this length of incubation, as shown in Figure 2, the most heavily labeled of slowly transported proteins (including actin and tubulin) become visible, as do the rapidly transported proteins.

The normal central nervous system synaptic targets for DRG neurons are spinal cord neurons, and, *in vitro,* spinal cord cells modify the direction of DRG neurite growth (Peterson and Crain, 1982) and can receive synaptic input from co-cultured DRG cells (Nelson et al., 1978). Interaction of the DRG neuronal axons with cultured spinal cord cells for 4 d prior to addition of ^{35}S-methionine to the cultures results in a significant reduction in the labeling of 3 prominently labeled proteins, of ap-

parent molecular weights of 32, 43, and 68 kDa (Fig. 3 and Table 1). By comparison, the labeling of actin and tubulin do not change (Fig. 3). The 3 regulated proteins are among the most heavily labeled of the rapidly transported group, as is shown in Figure 2. We did not rigorously pursue the possibility that other rapidly transported, but faintly labeled proteins are also affected, although visual inspection did not so suggest. It is possible, therefore, that changes in the labeling of these regulated proteins reflect a more general regulation of rapidly transported proteins. There is an increase in labeling of actin and tubulin (Table 1) that we interpret (see Discussion) as reflecting proportional diminution in the level of labeling of the other proteins.

In view of the prominence of these target-induced alterations, it was of interest to examine whether other tissues that receive innervation from the DRG *in vivo* exert a similar influence upon DRG axonal proteins *in vitro*. Muscle receives abundant sensory innervation from the DRG. Such nerves terminate on muscle spindles, Golgi tendon organs, and as free nerve endings within the muscle (for reviews, see Matthews, 1981, and Perl, 1984). DRG axonal contact with muscle cells in culture causes a significant reduction in the labeling of the 32 and 43 kDa proteins (Fig. 4; Table 1). There is also a decrease in the level of the 68 kDa protein, but it does not reach the level of significance by quantitation (Table 1).

It was also interesting to examine whether contact with DRG cells influences the level of these axonal proteins. DRG neurons have not been noted to synapse upon each other (Lieberman, 1976); thus such cells presumably should not serve as satisfactory targets for one another. However, non-neuronal cells of the DRG, at least in culture from chick, do interact with DRG neurons, thereby altering DRG neuronal morphology from bipolar to pseudounipolar (Mudge, 1984). DRG cultures, including both neuronal and non-neuronal cells, when plated in the axonal compartment, do not cause a significant diminution in the levels of the 32 or 68 kDa proteins, although there is a slight diminution in the 43 kDa protein by quantitation (Fig. 5; Table 1).

Finally, we investigated the effects exerted by skin fibroblasts that are not known to be targets of DRG neurons, and the effects of heart cells. Heart receives sensory innervation predominantly via the vagus, with a much smaller contribution from the DRG (Khabarova, 1961; Floyd, 1979). Neither of these tissues exerted a significant effect on any of the noted proteins (Fig. 5; Table 1).

Soluble factors, such as NGF, have been noted as being se-

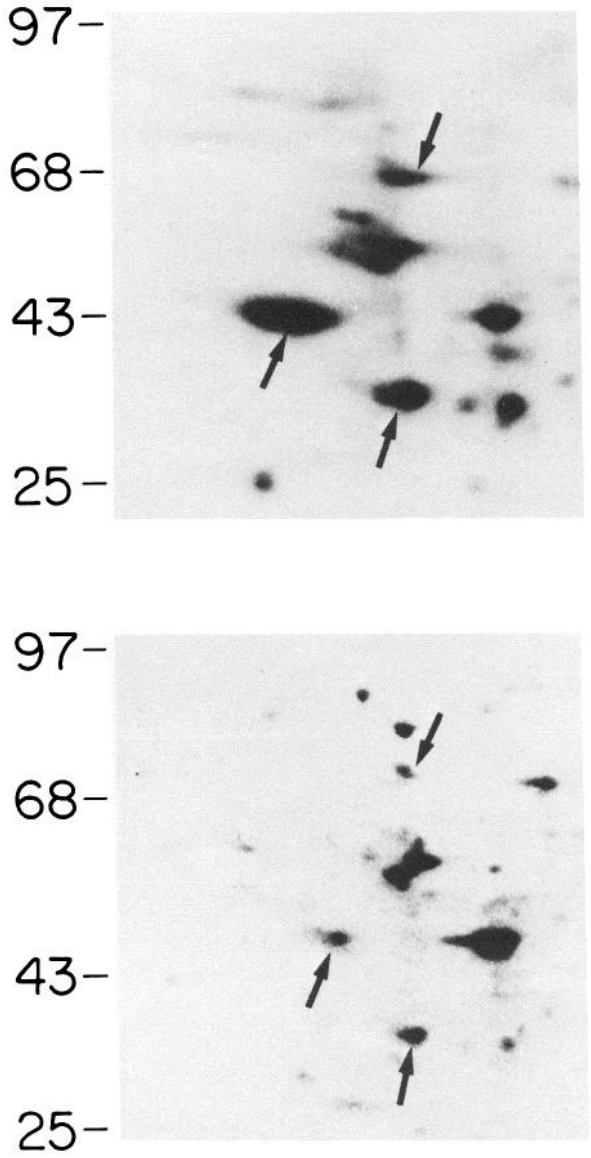

Figure 4. Effects of muscle cells plated as targets on rapidly transported DRG proteins. DRG neurons were either cultured alone (*upper panel*) or for 4 d with dissociated muscle cells plated as target (*lower panel*) prior to labeling. *Arrows* denote those proteins that consistently display diminished labeling following contact with muscle cells. These results were obtained in 6 separate experiments.

Table 1. Change in the level of the noted proteins with different targets

Spot	Sp cord	Muscle	DRG	Heart	Fibroblasts
68 kDa	0.51[a]	0.56	0.89	1.09	1.07
32 kDa	0.37[a]	0.33[a]	1.07	1.35	0.82
43 kDa	0.30[a]	0.27[b]	0.59[a]	0.51	0.88
Actin	2.39[b]	2.25[b]	0.77	1.14	1.27
Tubulin	1.83[b]	1.28	2.23	0.91	0.81

The densities for each spot were normalized, for each autoradiogram, to the total density of all spots on the gel. The mean normalized density for each type of target ± SEM was calculated for each spot and compared to the mean normalized density for the controls for the series. Presented here is the fraction, $(\text{mean normalized density})_{\text{target}}/(\text{mean normalized density})_{\text{control}}$. $N = 5$ for spinal cord, 6 for muscle, 4 for DRG, fibroblasts, and heart cells. Enclosed in heavy lines are those spots that diminished significantly in intensity during target contact.

[a] Test significantly different from control, $p < 0.05$.

[b] Test significantly different from control, $p < 0.01$.

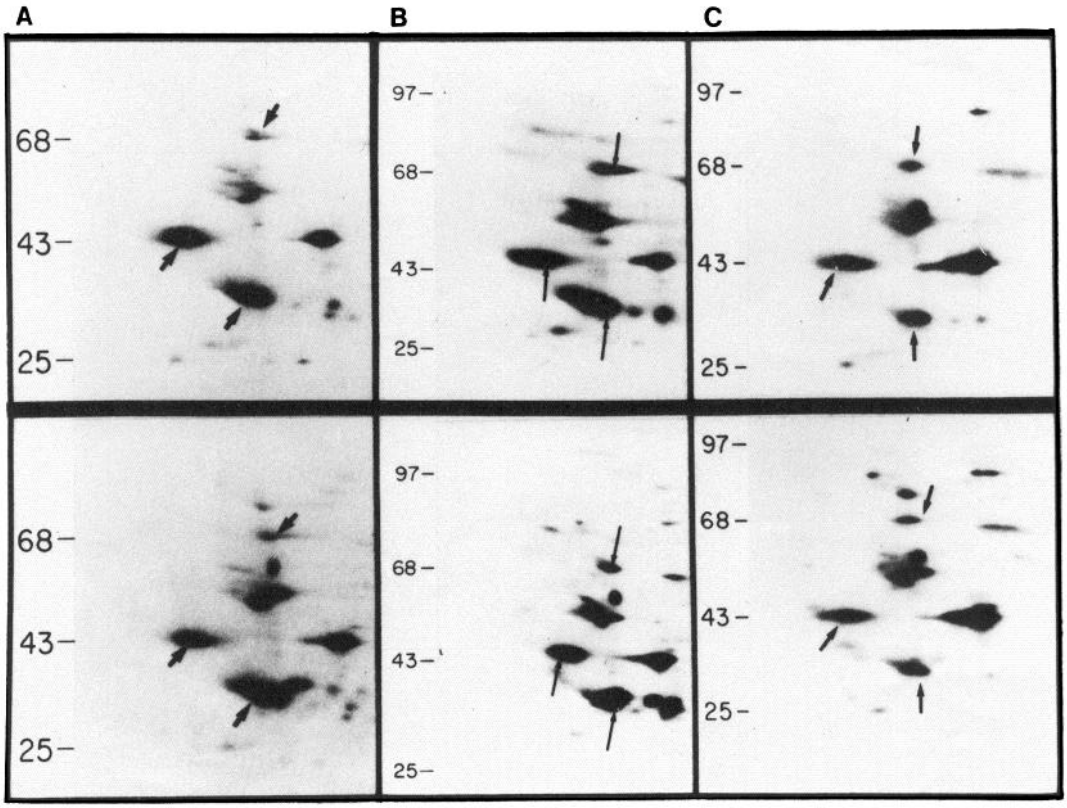

Figure 5. Several cell types do not affect the noted rapidly transported proteins, or do so only minimally. These include DRG cells (*A*), skin fibroblasts (*B*), and heart cells (*C*). In each case, DRG cells were either plated alone (*upper panel*) or with one of the 3 types of target cells (*lower panel*). These results were obtained in 4 independent experiments for each of the target tissues.

creted by target cells (Barth et al., 1984) and as influencing neurite growth and protein expression in responsive neurons (Greene and Shooter, 1980; Harper and Thoenen, 1980). Medium continuously conditioned by spinal cord cells does not influence these proteins (Fig. 6). This suggests that this regulation is exerted either by direct contact between spinal cord cells and DRG axons or by extremely labile secreted factors.

One of these regulated axonal proteins (molecular weight about 43 kDa) displays electrophoretic mobility similar to that of GAP-43, an axonally transported protein previously noted as being expressed at elevated levels during periods of neuronal growth and regeneration (Skene and Willard, 1981a, b; Skene, 1984). Western blot analysis of DRG axonal proteins, using an anti-GAP-43 antibody provided by J. Freeman, reveals that the 43 kDa spot does include immunoreactive GAP-43 (Fig. 7).

Discussion

Specificity of target recognition

This work provides evidence that even in cell culture, the growing DRG neurons can distinguish between different classes of cells, and that the recognition of particular cells is accompanied by specific alterations in the protein constituency of the DRG neurons. It is most clear-cut that spinal cord cells cause a reduction in the rapidly transported proteins and that fibroblasts or heart cells do not. Muscle cells, as an example of peripheral target tissue, also cause a significant diminution in the labeling of the 32 and 43 kDa proteins, but the reduction in the 68 kDa does not reach the level of statistical significance. Like fibroblasts and heart, cells of the DRG cells leave the 68 and 32 kDa unaffected. They do cause a slight reduction in the 43 kDa protein. One explanation for such a differential effect is that the various proteins are involved to different degrees in distinct facets of the growth or recognition processes. However, we do not believe our quantitation adequate for making such subtle distinctions.

The metabolic site of regulation of these proteins is not identified here; it could be at the level of synthesis, transport, degradation, or secretion. However, the 3 target-regulated proteins serve as signatories to a specific recognition event whereby DRG neurons may distinguish target from non-target. This is compatible with the ample morphological and physiological evidence that demonstrates that neurons ramify and maintain processes preferentially within target tissue both *in vivo* (e.g., Landmesser, 1980; Easter et al., 1985) and *in vitro* (Bonhoeffer and Huf, 1982; Crain and Peterson, 1982), and that some proteins in regenerating goldfish optic nerve manifest regulation through contact with the optic tectum (Benowitz et al., 1983).

We have interpreted the changes as diminutions in the levels of the 3 rapidly transported proteins rather than as increases in the levels of actin and tubulin, although the autoradiographic picture of these 2 alternatives would be indistinguishable. We believe that the level of actin and tubulin remains stable. Our evidence derives from prior investigations designed to investigate both slowly and rapidly transported proteins by the use of longer labeling periods (Sonderegger et al., 1983). Under those conditions, the levels of actin and tubulin were not noted to change.

We are at present attempting to identify the regulated proteins. As described, the regulated 43 kDa protein is, or includes, immunoreactive GAP-43. This is of especial interest in light of

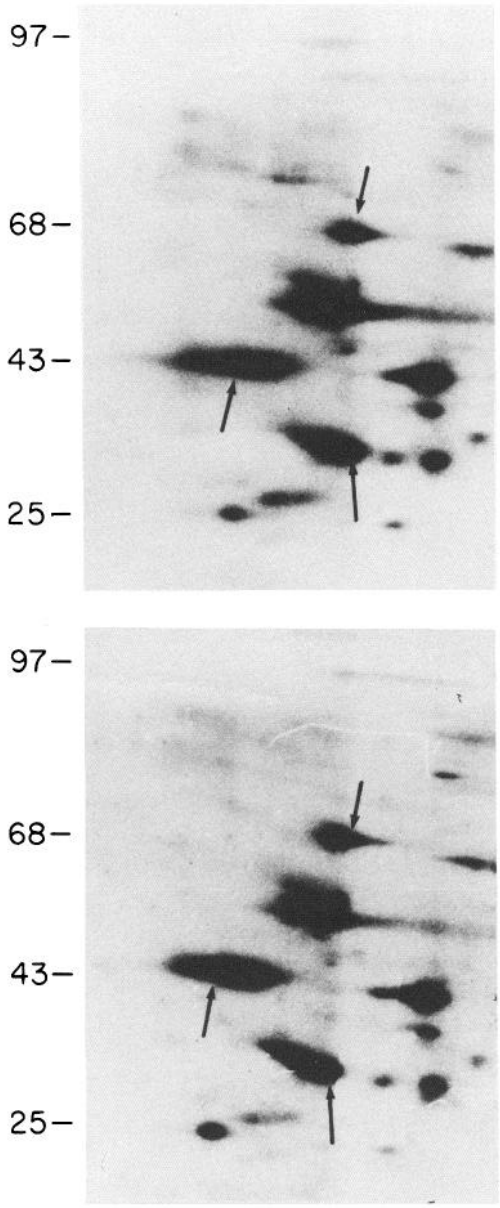

Figure 6. Soluble factors released from cultured spinal cord cells do not affect the levels of the noted proteins. DRG neurons grown in control spinal cord medium (see Materials and Methods) (*top panel*) or in the presence of continuously conditioned medium (*bottom panel*) do not manifest significant differences in the level of proteins noted to be regulated by contact with spinal cord cells (*arrows*). For these experiments, spinal cord cells were plated within circular Teflon chambers fastened tightly to the dish in a manner that prevented axonal egress or ingress. The height of the chamber was low to allow complete exchange of medium with the DRG and their processes. An identical number of spinal cord cells were plated as in the experiments in which spinal cord cells were used as targets. This result was obtained in 5 separate experiments.

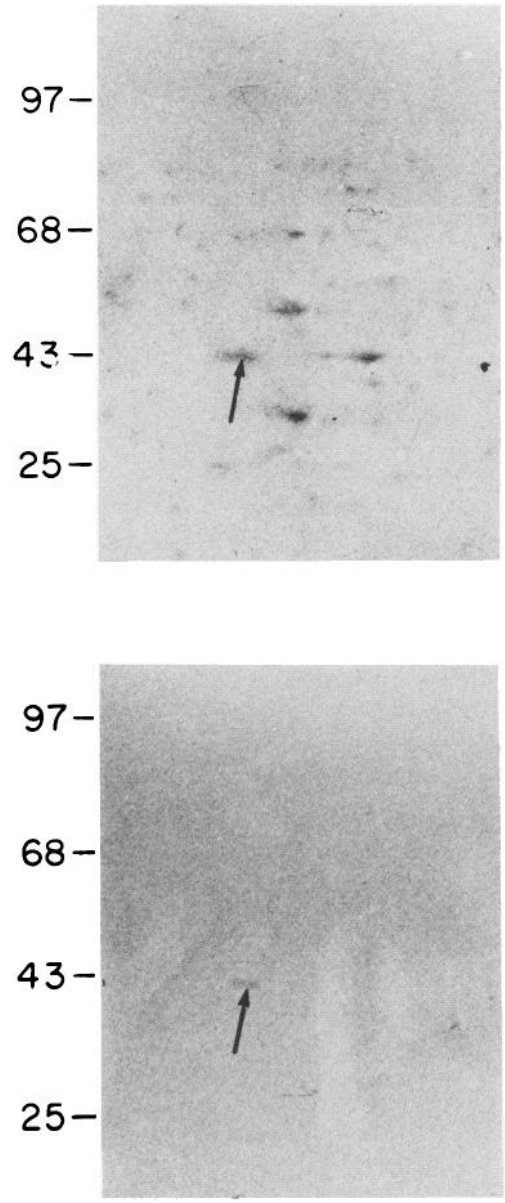

Figure 7. Western blot analysis of the 43 kDa regulated axonal protein demonstrates antigenic cross-reactivity with GAP-43. DRG axonal proteins were labeled with ^{35}S-methionine, resolved by 2-dimensional electrophoresis, and electrophoretically transferred to nitrocellulose. Proteins were visualized by autoradiography (*upper panel*). The same sheet of nitrocellulose was incubated overnight with polyclonal rabbit anti-GAP-43 antiserum and bound immunoglobulin visualized with horseradish peroxidase-conjugated goat anti-rabbit antiserum (*lower panel*). The only axonal protein that is labeled by this antibody has, as shown by the *arrows*, electrophoretic characteristics identical to the regulated 43 kDa protein.

recent observations that GAP-43 is a major component of growth cones (Meiri et al., 1986; Skene et al., 1986). This work cannot definitively establish whether the level of GAP-43 is target-regulated. Recent cloning of cDNA for GAP-43 does provide evidence of regulation at the level of gene expression, with an increase in GAP-43 RNA level during periods of neurite growth (Karns et al., 1987).

The prominence of the down-regulation of the 3 proteins suggests that many of the DRG neurons have been influenced by the targets, especially of the spinal cord. An individual DRG neuron may therefore be capable of interacting with a variety of tissues from several locales, despite the usual *in vivo* restriction of its central terminals to a somatographically restricted region of the spinal cord, and of its peripheral terminals to a specific receptive field. In culture, or after transplantation, neurons may innervate targets that include tissues or regions other than those normally innervated *in vivo* (Puro et al., 1977; Stanfield and O'Leary, 1985), although significant specificity may also be retained (Peterson and Crain, 1982; Camardo et al., 1983). DRG neurons in culture innervate spinal cord cells in

their proximity without obvious bias (Nelson et al., 1978), and when thoracic ganglia in tadpoles are transplanted to more cranial regions, they innervate muscle spindles and ventral spinal cord, structures and regions not normally their targets (Smith and Frank, 1985). This suggests that a variety of potential target tissues are recognized by most or all sensory neurons of the DRG as distinguished from non-target tissues.

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