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Quantitative analysis of GAP-43 expression by neurons in microcultures using cell-ELISA

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Abstract

A cell-ELISA technique is described which allows the quantification of GAP-43 protein in a large number of microcultures of adult dorsal root ganglion neurons. GAP-43 is measured in the 1–10 ng range, corresponding to the amount of GAP-43 present in fewer than 500 DRG neurons. Specificity of the assay is confirmed using Western blotting and immunocytochemistry. The GAP-43 content of adult DRG microcultures rises during 2 weeks in culture, although the number of surviving neurons decreases. The GAP-43 content of cultured adult DRG neurons is not increased by chronic exposure to added nerve growth factor after 7 days in vitro. However, GAP-43 is increased in DRG taken from animals with prior peripheral nerve injury, and is decreased by chronic exposure to dibutyryl cyclic AMP after 7 days in vitro. The method affords the sensitivity and statistical power to document modest changes in GAP-43 protein abundance in complex cultures. © 1997 Elsevier Science B.V.

Keywords: cAMP; Dorsal root ganglion; ELISA; GAP-43; NGF; Regeneration

1. Introduction

Antibodies have been used to study the changes in GAP-43 expression which occur in adult dorsal root ganglion (DRG) neurons following axonal injury in situ using both a qualitative Western blot approach (Schreyer and Skene, 1991), and a quantitative immunocytochemical approach (Schreyer and Skene, 1993). In order to further examine the mechanisms which control the response of neurons to injury, we are studying the expression of GAP-43 in cultures of dissociated adult DRG tissue. Although densitometric quantification of GAP-43 immunoreactivity on Western blots is one possible approach to estimating GAP-43 protein content of DRG cultures, we have found this method to be cumbersome and inconsistent. We have therefore sought to develop a more accurate, more efficient method for the analysis of GAP-43 protein in large numbers of different cultures.

Enzyme-linked immuno-sorbent assays of antigens present in cell cultures (commonly called 'cell-ELISA' or 'CELISA') have been in use for over a decade (for review, see Sedgwick and Czerkinsky, 1992), but primarily to examine cell surface antigens. GAP-43 is an internal, membrane bound, largely hydrophilic protein which is variably expressed in cells of the nervous system (for review see Skene, 1989). Thus, any GAP-43

Abbreviations: dbcAMP, dibutyryl cyclic adenosine monophosphate; DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; GAP-43, growth-associated protein 43Kd; MAP2, microtubule-associated protein 2; NGF, nerve growth factor; NMS, non-immune mouse serum; OPD, *o*-phenylenediamine dihydorochloride; PBS, phosphate buffered saline.

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cell-ELISA protocol has to meet the somewhat conflicting requirements that the cells in culture be permeabilized, that the GAP-43 be 'captured' (bound to the solid phase), and that antigenic recognition sites be preserved.

Monoclonal antibody 9-1E12 was chosen for the development of this assay because it is known to recognize an epitope present on all forms of GAP-43 (Schreyer and Skene, 1991), because it is extremely sensitive, and because it provides excellent immunocytochemical labeling of neurons in tissue culture (Goslin et al., 1988, 1990). A significant limitation of the utility of this antibody is that it fails to recognize GAP-43 from non-mammalian species.

We report here a quantitative cell-ELISA for GAP-43 in adult rat DRG cells grown in microculture in 96-well microtiter plates. The assay employs monoclonal antibody 9-1E12 and a horseradish peroxidase-conjugated secondary antibody. It reliably produces a colorimetric signal related to GAP-43 abundance in the range of 1–10 ng GAP-43 protein per microwell. In order to accurately interpret the results obtained with this procedure, we analyze sister cultures with Western blotting to assure that the 9-1E12 immunoreactivity present in cultures can be identified as authentic GAP-43, with immunocytochemistry for GAP-43 to identify which cells contain the immunoreactivity, and with immunocytochemistry for MAP-2 to identify and count neurons to determine their survival rate.

2. Materials and methods

2.1. Adult DRG cell culture

Adult rat DRG cultures were established in plastic 96-well microtiter plates (Nunc; # 16805) for cell-ELISA and immunocytochemistry, or plastic 24-well plates (Sarstedt; # 83.1836) for Western blotting. In each case, the culture wells were first coated with a 0.1 mg/ml solution of poly-L-lysine (Sigma; P-9155) overnight at 37°C. The wells were then rinsed four times with sterile distilled water and once with culture medium (see below) just before use.

Dissociation of adult DRG tissue was performed using a variation of the method of Lindsay (1988). DRG were dissected from 175–200 g female rats euthanized by exposure to carbon dioxide. Approximately 40–48 DRG were obtained from each rat and held in L15 medium (Sigma; L-4386). In some cases rats were prelesioned by resecting the sciatic nerve at mid thigh level 3 days prior to euthanasia. Only the L4, L5, and L6 DRG on the ipsilateral and contralateral sides were dissected from pre-lesioned rats. The DRG were cleaned of connective tissue, minced with # 11 scalpel blades and rinsed again in L15. The tissue was resuspended in 5 ml 0.1% collagenase (Gibco BRL; # 17018-029) in L15 for 90 min at 37°C, with agitation every 20–30 min. The tissue was then briefly triturated through a fire polished, serum coated pasteur pipet until it was partially disrupted (8–10 passes). The partially dissociated tissue was washed once by centrifugation (200 × g, 10 min) in L15, then resuspended in 5 ml 0.02% trypsin (Sigma; T-0646) in L15. The suspension was incubated at 37°C for a further 30 min, with occasional aggitation. Trypsin activity was quenched by the addition of 0.5 ml of horse serum. Dissociation was completed by trituration through a fire polished, serum coated, pasteur pipet, and the cells were washed once by centrifugation in F14 medium (Gibco BRL; Formula 92-5138EA) and 10% horse serum (locally produced). The cells were resuspended in 2 ml F14 and 10% horse serum and the number of viable neurons was counted, in the presence of 0.06% nigrosin (Sigma; N-4763) using a hemocytometer. The desired number of neurons were suspended in F14 and 10% HS, and plated. Cultures were seeded at a density of 500 neurons per well in 96-well plates or 5000 neurons per well in 24-well plates. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Half of the medium was aspirated and replaced with fresh medium on the following day, then every 2 days thereafter. Adult DRG neurons cultured using this technique are not separated from non-neuronal cells. Thus, in these cultures, the non-neuronal DRG cells (particularly Schwann cells) survive and proliferate.

2.2. Medium supplements

In some experiments the DRG culture medium was supplemented with 100 ng/ml nerve growth factor (NGF) (Cedarlane Laboratories; McNet-001) or a sheep antibody to mouse NGF (Cedarlane Laboratories; McNet-041) diluted to 2 μg/ml, or with both. In a separate series of experiments 0.5 mM dibutyryl adenosine 3′,5′ cyclic monophosphate (dbcAMP) (Sigma; D-0627) was added to the medium. These supplements were included in the medium at the time of plating, and were renewed each time the cultures were fed.

2.3. Standard curves

GAP-43 was partially purified from postnatal day seven rat brains by preparative isoelectric focusing (Radola, 1984). The amount of GAP-43 in these preparations was measured by densitometric scanning of the GAP-43 band in Coomassie stained gels, compared to a bovine serum albumin (Sigma; A-2153) standard. Total protein was estimated by the method of Lowry et al. (1951).

Partially purified GAP-43 (0–11 ng GAP-43 protein) was diluted in 50 μl of distilled water and added to wells of the same 96-well microtiter plates used for tissue culture, but without prior coating with poly-L-lysine. Dilute protein solutions were handled in microfuge tubes pretreated with Western blot blocking solution (see below) to prevent adsorptive loss of the protein. The total protein added was well below the protein binding capacity of the plastic (approximately 100 ng/well). Added protein solution was allowed to bind overnight at 37°C. At the end of the binding period, approximately 10% of the applied GAP-43 could still be detected in the protein solution by Western blotting, or by rebinding in fresh wells (not shown), suggesting that GAP-43 binding was not complete. Quantitation of GAP-43 in these studies should therefore be regarded as an estimation, rather than as an absolute determination. Wells containing GAP-43 standards were processed in parallel with the wells containing cultured cells (see below).

2.4. Fixation and processing of cultures and standards

The culture medium was removed by gently upending the plate over a sink, and the wells were rinsed once with phosphate buffered saline (PBS). The wells were then fixed in 100% methanol at −20°C for 30 min. The methanol was removed and the plates were dried for an additional 30 min under vacuum at room temperature. This fixation method allows virtually complete retention of cells through all subsequent steps. Each well was filled with 400 μl of a blocking solution consisting of 10% nonfat dry milk and 0.05% Tween 20 (Sigma; P-1379) in PBS (insoluble material removed by centrifugation) for 1 h at 37°C. This was then replaced with 50 μl of blocking solution containing monoclonal antibody 9-1E12 ascites fluid (Schreyer and Skene, 1991), diluted 1:5000. In control experiments, primary antibody was replaced with non-immune mouse serum (NMS) diluted 1:500–1:5000, or it was omitted altogether. Incubation with primary antibody was carried out for 45 min at room temperature. All wells were washed three times for 5 min with PBS. The wells were then filled with 50 μl of a secondary antibody, horseradish peroxidase conjugated anti-mouse IgG (Sigma; A-2304) diluted 1:500 in blocking solution. Incubation with secondary antibody was carried out for 30 min at room temperature. All wells were again washed three times for 5 min with PBS. Peroxidase activity was quantified by adding 200 μl of water containing the substrates o-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide, supplied in tablet form (Sigma; P-9187) and diluted according to the manufacturer's instructions. The reaction was carried out for 30 min at room temperature, then stopped by the addition of 50 μl of 3 M hydrochloric acid. The absorbance of the solution in each well was measured (absorbance at 490 nm minus absorbance at 595 nm) using a Molecular Devices V_{max} microplate reader, using wells without cells or GAP-43 protein, but processed with all antibodies, as blanks. Standards were usually assayed in duplicate; cultures were assayed in sextuplicate.

2.5. Immunocytochemistry

Cultures to be used for immunocytochemistry were rinsed in PBS, fixed in methanol at −20°C for 30 min, dried under vacuum, then treated with a blocking solution consisting of 2% horse serum and 0.5% bovine serum albumin in PBS for 1 h. This was then replaced with either anti-GAP-43 monoclonal antibody 9-1E12 ascites fluid diluted 1:5000, or anti-MAP2 monoclonal antibody AP-20 (Sigma; M 1406) diluted 1:200 in blocking solution for 45 min. After washing three times for 5 min with PBS, the wells were treated with biotinylated anti-mouse IgG (Sigma; B-7151) diluted 1:500 in blocking solution for 30 min. The wells were washed again three times for 5 min with PBS, then treated with avidin-peroxidase (Sigma; A-3151) diluted 1:500 in blocking solution for 30 min. The wells were washed again three times for 5 min with PBS, then reacted with 0.025% diaminobenzidine (DAB) (Sigma; D-5637) and 0.05% hydrogen peroxide (Fisher; H325-100) in PBS for 20 min, and washed again with PBS. For neuron counting and photography the wells were filled to the brim with buffer, capped with a cover slip and examined using Koehler illumination or phase contrast optics on a Zeiss Axiovert 100 inverted microscope.

2.6. Western blotting

Cultures to be used for Western blot analysis were prepared in 24-well plates (5000 neurons/well) and grown for 2–14 days. Unfixed cultures were rinsed once in PBS at 37°C, then solubilized in 500 μl/well of 1% SDS at 37°C. The SDS protein extract was pooled from two wells at each survival time, and stored frozen. A sample of the original dissociated cell preparation (10 000 neurons) was also solubilized in SDS and stored frozen. Each entire protein preparation (corresponding to 10 000 neurons initially plated) was electrophoresed on a 12% acrylamide gel (Laemmli, 1970), then electroblotted to nitrocellulose (Schleicher and Schuell; BA-S 83) in 40 mM AMPSO (Sigma; A-6659) plus 20% methanol, pH 9.5. Blots were treated with a blocking solution of 2% horse serum and 0.5% bovine serum albumin in PBS for 30 min. This was replaced with 9-1E12 ascites fluid diluted 1:2000 in blocking solution for 45 min. After washing with PBS, the blots were developed using biotinylated anti-mouse IgG (Sigma; B-7151) followed by avidin linked peroxidase (Sigma;

A-3151), each diluted 1:5000 and applied for 30 min. Peroxidase activity was revealed by reacting with 0.025% diaminobenzidine and 0.05% hydrogen peroxide for 30 min.

3. Results

3.1. Useful range of assay

In order to determine the useful quantification range of the assay, microtiter wells, which had been coated with purified GAP-43 protein were studied. Initial experiments indicated that GAP-43 protein was better retained on standard, uncoated polystyrene plates than on poly-L-lysine coated polystyrene, or on polyvinyl chloride. Wells coated with partially purified GAP-43 displayed an increasing relationship between antigen amount and signal strength (Fig. 1). The curve was steeper if higher concentrations of primary or secondary antibody were used (not shown).

In the lower concentration range, the binding curve for GAP-43 standards deviated significantly from linearity, possibly reflecting cooperativity in the binding of bifunctional immunoglobulins. The curve between 0 and 8–10 ng GAP-43 was fitted with a second order regression using SigmaPlot software (Fig. 1). Above 8–10 ng GAP-43 per well, the rate of rise of absorbance with increasing applied GAP-43 began to decrease, and a saturating signal was obtained at approximately 2.5–3.0 absorbance units (not shown). It is not known if saturation was governed by the limit of binding of antigen to the plastic, saturation of antibody-antigen binding, the limit of the substrate reaction or the limit of the spectrophotometric instrument.

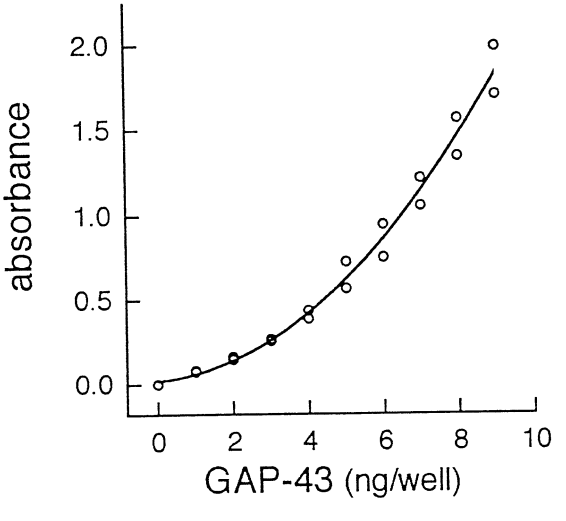

Fig. 1. GAP-43 standard curve for cell-ELISA. Partially purified GAP-43 protein was applied to wells of a 96-well plate and analysed by ELISA. Each point was determined in duplicate. Absorbance ($A_{490}-A_{595}$) was measured for each well, and a best fit curve was calculated using second order linear regression analysis. Similar standard curves were obtained in parallel with every culture experiment.

3.2. Background

When partially purified GAP-43 protein was used as an antigen, omission of either the antigen, the primary antibody, or the secondary antibody resulted in an absorbance reading which was not significantly different from that of the substrate solution alone (not shown). Wells containing no cells and no purified GAP-43 were used as spectrophotometric 'blanks' and their average reading was assigned a value of zero. In cell-free assays, substitution of NMS for the primary antibody at dilutions of 1:500 or greater resulted in no signal detectable above background (not shown).

Similarly, wells containing cell cultures were processed as negative controls. Omission of either the primary or secondary antibody, or substitution of NMS for the primary antibody at dilutions of 1:2000 or greater resulted in no signal detectable above background (not shown), indicating that endogenous peroxidase activity and non-specific antibody binding were acceptably low. The cultured cells alone had no detectable absorbance.

3.3. Specificity

In cultures prepared for GAP-43 immunocytochemistry using antibody 9-1E12 (Fig. 2A), strong labelling of virtually all neurons was present after 7 days in vitro. Immunoreactivity was found in the perinuclear cytoplasm and throughout the extensive length of the neurites. Neurite labelling usually had a beaded appearance, but the growth cones at the tips of neurites were more evenly labeled.

Schwann cells and other non-neuronal cells in whole DRG cell cultures displayed no detectable GAP-43 immunoreactivity in immunocytochemical preparations, but cultures of purified adult rat Schwann cells prepared by the method of Brockes et al. (1979) could display immunocytochemical labelling with our GAP-43 antibody (not shown). However, when GAP-43 cell ELISA was performed on microcultures containing a confluent monolayer of purified Schwann cells, the values obtained were always $\leq 15\%$ of the values obtained from whole DRG cultures processed in parallel (not shown).

Adult DRG neurons in our cultures were easily recognized under phase contrast optics because of their large, round somata (Fig. 2B and D). Neuronal somata were strongly labelled by the MAP2 immunocytochemistry procedure (Fig. 2C). In some neurons, MAP2 labelling extended a short distance along some neurites. Non-neuronal cells displayed low, but detectable, MAP2 immunoreactivity. MAP2 immunocytochemical

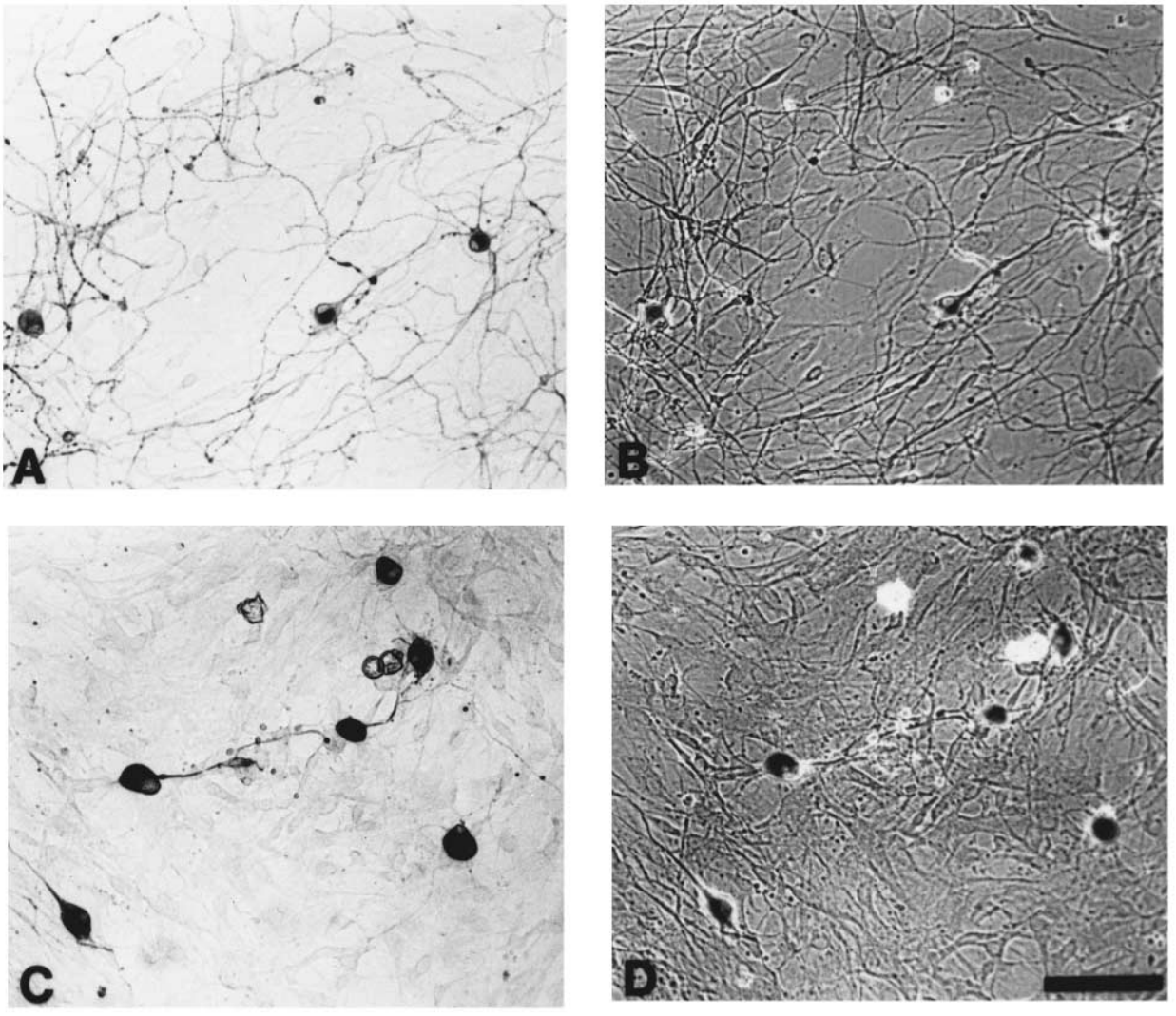

Fig. 2. Microscopic appearance of adult rat DRG cultures after 7 days in vitro. A. GAP-43 immunocytochemistry with (B) corresponding phase contrast image, (C) MAP2 immunocytochemistry with (D) corresponding phase contrast image. Bar indicates 100 μm.

preparations proved to be much easier than simple phase contrast for rapid counting of neurons.

Antibody 9-1E12 labelled a single band on Western blots prepared from DRG cell cultures (Fig. 3). This band co-migrated with GAP-43, purified from neonatal rat brain. The intensity of the 9-1E12 labelled band increased with increasing time in culture.

3.4. Time course of GAP-43 accumulation in DRG cultures

To evaluate the changes in GAP-43 protein in DRG cell cultures over time, one DRG preparation was used to seed wells on five different plates. After 2, 4, 7, 10 or 14 days in vitro, GAP-43 ELISA and MAP2 immunocytochemistry were carried out on separate wells of each plate. We have previously found that in cultures shorter than 2 days, the ELISA cannot be performed because of poor neuronal adhesion to the substratum.

To compare the results from assays performed on different days, each plate also contained a standard curve of partially purified GAP-43. Second order regression equations from these standard curves were used to calculate the amount of GAP-43 in each culture well. The total amount of GAP-43 detected per well increased more than three-fold (Fig. 4a) from 2 to 14 days in vitro.

MAP2 immunocytochemistry (Fig. 2B) was used to identify neurons in sister DRG cultures as a means of evaluating neuronal survival, independent of GAP-43 content. All MAP2 immunopositive neurons in each well were counted. The number of surviving neurons decreased with increasing time in vitro, so that approximately 60% of plated neurons survived at 2 days, and approximately 40% survived at 10 days (Fig. 4B). At 14 days in vitro, neurons and non-neuronal cells were clumped and difficult to count, likely resulting in under-

estimation of survival. When changing neuronal survival is accounted for, the amount of GAP-43 present per surviving neuron underwent a greater than four-fold increase from 2 to 10 days in vitro.

3.5. Regulation of GAP-43 abundance in adult DRG cultures

In subsequent experiments, raw absorbance values were converted to GAP-43 content using second order regression equations from standard curves included on the same plate, then adjusted for neuronal survival based on mean counts of MAP2 immunopositive neurons in identical sister cultures. Data were expressed as GAP-43 immunoreactivity per neuron, normalized to control wells.

Adult DRG cell cultures were examined for GAP-43 content after 7 days in vitro in control medium, in medium with 100 ng/ml NGF added, in medium with 2 μg/ml blocking antibody to NGF added, or in medium with both NGF and anti-NGF added (Fig. 5). Neither the neuron survival rate nor the GAP-43 immunoreactivity were significantly changed by addition of NGF. Addition of blocking antibody to NGF caused a 12.8% decrease in neuronal survival (not shown), possibly reflecting the existence of a small NGF-dependent subpopulation of adult DRG neurons. Anti-NGF caused a small, but significant ($P < 0.05$) increase in GAP-43 immunoreactivity per neuron. These effects of anti-NGF seemed to be partially reversed by including added NGF.

Cultures derived from adult lumbar DRG were examined for the effect of prelesioning the sciatic nerve 3 days prior to removal for tissue culture. The survival rate of prelesioned DRG neurons was 31.6% higher (not shown), possibly reflecting selection for an injury-resistant population. After 7 days in vitro the amount of GAP-43 immunoreactivity per neuron in prelesioned DRG cultures was approximately 30% greater ($P < 0.01$) than the already elevated amount seen in control cultures (Fig. 6).

Chronic addition of dbcAMP caused a small decrease in neuronal survival, and a large decrease in GAP-43 immunoreactivity. After adjustment for neu-

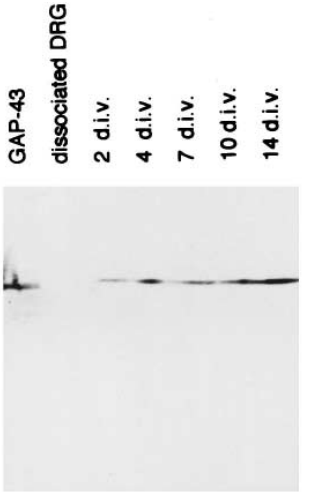

Fig. 3. Western blot for GAP-43 probed with antibody 9-1E12. First lane includes partially purified preparation containing 100 ng GAP-43. Second lane includes SDS extract of frozen, dissociated DRG cell preparation (10 000 neurons). Remaining lanes include SDS extracts of adult rat DRG cultures (initial seeding of 10 000 neurons) after 2, 4, 7, 10, or 14 days in vitro.

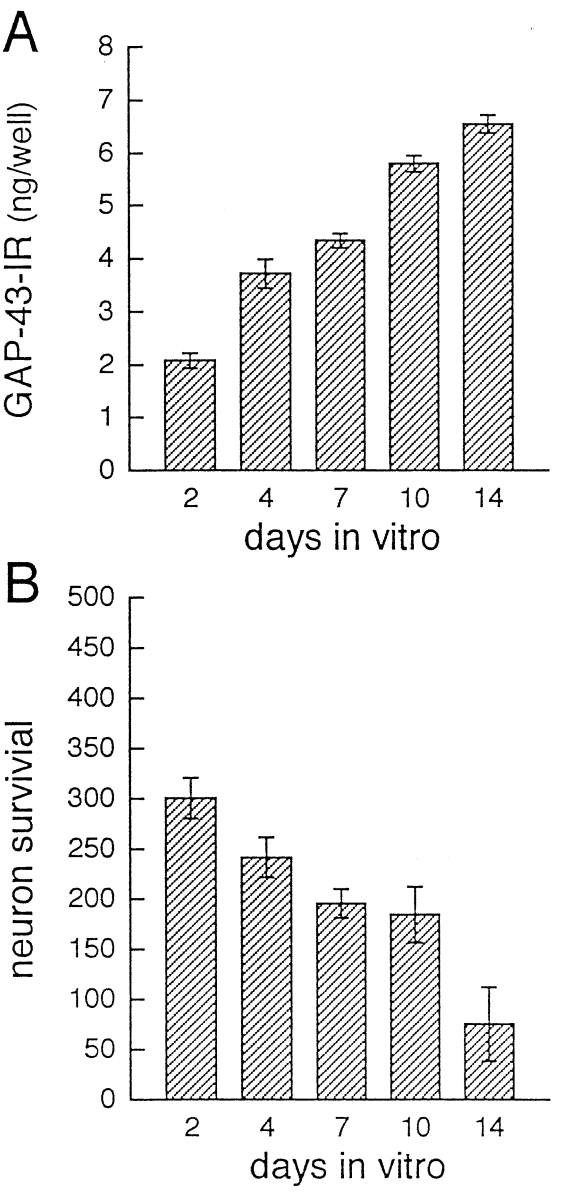

Fig. 4. A. GAP-43 ELISA of adult rat DRG cultures after 2, 4, 7, 10 and 14 days in vitro, expressed as ng GAP-43 per well. Each determination was made in sextuplicate. B. Neuron counts of sister adult rat DRG cultures, expressed as MAP2 immunopositive neurons per well. Each determination was made in quadruplicate. Error bars indicate standard error of the mean. Results are representative of three experiments.

ronal survival, the decrease in the GAP-43 content of adult DRG cultures containing dbcAMP was found to be significant, whether in cultures derived from lesioned ($P < 0.01$) or unlesioned ($P < 0.01$) DRG (Fig. 6).

4. Discussion

4.1. GAP-43 cell ELISA

Standard culture methods have been adapted for use in a 96-well plate format as a means to provide rapid analysis of large numbers of individual cultures. A cell-ELISA method has been devised that both exposes and preserves GAP-43 antigenic sites in fixed neurons, which remain attached to the culture substratum. The use of a peroxidase reporter enzyme and the substrate OPD (which forms a soluble, colored reaction product) facilitates the quantification of immunoreactivity by allowing absorbance measurements to be made in the original culture plates using a plate reader.

Under our assay conditions, we observed no non-specific binding of immunoglobulin from non-immune mouse serum at concentrations up to 40 times higher than the concentration of 9-1E12 ascites fluid used. When the 9-1E12 antibody was used to probe Western blots of the proteins solubilized from mixed DRG cell cultures, only a single band was observed, corresponding to authentic GAP-43. We conclude that antibody

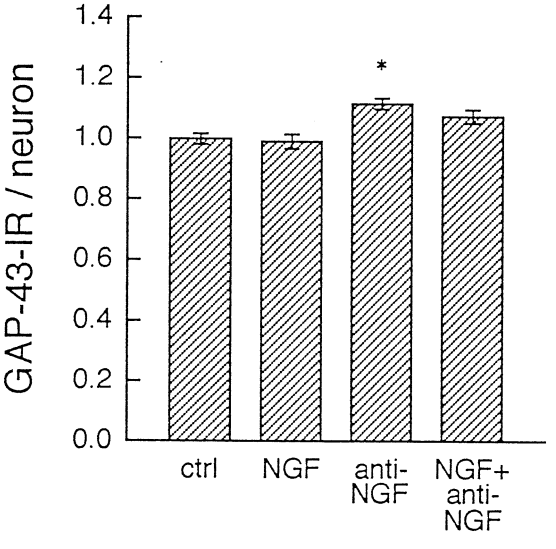

Fig. 5. GAP-43 ELISA of adult rat DRG cultures after 7 days in vitro. Experimental cultures were chronically exposed to 100 ng/ml NGF, 2 μg/ml antibody to NGF, or both. Determinations were made in sextuplicate and normalized to control and adjusted for neuronal survival (mean of four determinations) in identical sister cultures. Error bars indicate standard error of the mean. One way ANOVA indicates significant difference between groups ($P < 0.001$). Asterisk indicates significantly different from control (Student's t-test; $P < 0.05$). Results are representative of five experiments.

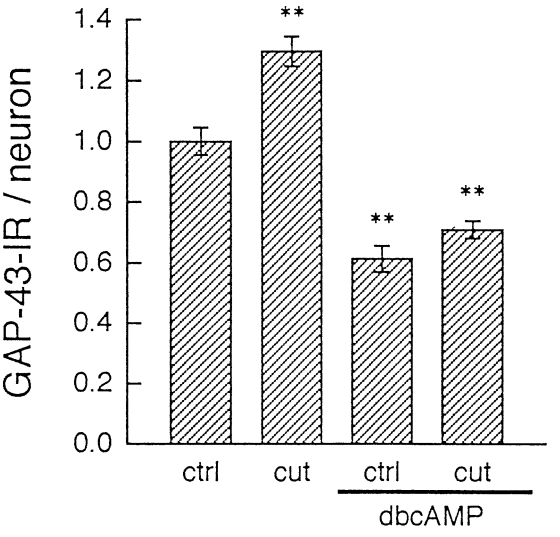

Fig. 6. GAP-43 ELISA of adult rat DRG cultures from a 3 day pre-lesioned rat, after 7 days in vitro. Cultures from lumbar DRG contralateral to a sciatic nerve resection are indicated by 'ctrl'; 'cut' indicates cultures from lumbar DRG ipsilateral to a sciatic nerve resection. Bar indicates cultures that were chronically exposed to 0.5 mM dbcAMP. Determinations were made in sextuplicate and normalized to control and adjusted for neuronal survival (mean of six determinations) in identical sister cultures. Error bars indicate standard error of the mean. One way ANOVA indicates significant difference between groups ($P < 0.001$). Double asterisk indicates significantly different from control (Student's t-test; $P < 0.01$). Results are representative of three experiments.

9-1E12, at the dilutions used in the cell-ELISA method, binds in a manner attributable to the specific recognition of GAP-43 by the primary antibody.

Although the assay can be used to demonstrate an increasing photometric signal with increasing added GAP-43, this relationship is not first order (Fig. 1). The GAP-43 antibody used in this study has not been proteolytically cleaved and maintains its bifunctional antigen binding structure. It would therefore be expected to display cooperative binding kinetics. We used a second order regression to fit the standard curves in the time course experiments, then used these equations to calculate absolute GAP-43 quantitites in all cultures. By expressing the signal as ng GAP-43, assays performed on different days could be directly compared to each other. For simplicity, the GAP-43 content values from subsequent 7 day cultures were normalized to control wells, then normalized again for the number of surviving neurons in identical sister cultures.

4.2. Cellular localization of GAP-43

GAP-43 protein can be found in Schwann cells under certain circumstances (Tetzlaff et al., 1989; Curtis et al., 1992; Scherer et al., 1994). In our studies, GAP-43 was not detected immunocytochemically in the non-neuronal cells contained in mixed DRG cell cultures. How-

ever, GAP-43 immunoreactivity could be seen in purified Schwann cell cultures (not shown). Studies in vivo suggest that Schwann cell expression of GAP-43 may occur when contact with neurons is removed (Tetzlaff et al., 1989; Young et al., 1992; Scherer et al., 1994). Quantification of GAP-43 immunoreactivity in purified Schwann cell cultures, as measured by ELISA, indicated that even when GAP-43 was expressed by Schwann cells, it was much less than in comparable cultures containing DRG neurons. We conclude that in cultures in which neurons are present and Schwann cell GAP-43 is presumably decreased even further, the vast majority of the GAP-43 immunoreactivity resides in the neurons.

4.3. Regulation of GAP-43 expresion in DRG neurons

GAP-43 is strongly expressed by all neurons during their initial development (McGuire et al., 1988; De la Monte et al., 1989; Dani et al., 1991; Reynolds et al., 1991), indicating that high levels of GAP-43 may be an early, inherent feature of neurons. With maturation, GAP-43 expression generally declines (Jacobsen et al., 1986; Basi et al., 1987). Although some adult DRG neurons retain moderate levels of GAP-43 expression (Schreyer and Skene, 1991, 1993; Stewart et al., 1992), peripheral axotomy causes GAP-43 up-regulation across the entire population which suffers injury (Verge et al., 1990; Schreyer and Skene, 1993). It has been hypothesized that uninjured adult DRG neurons are under the influence of a 'GAP-43 repressor' derived from peripheral tissues, and that upregulation following peripheral axotomy is due to removal of this repressor (Bisby, 1982; Schreyer and Skene, 1993). The present study indicates that NGF, which could possibly be derived from the non-neuronal cells of peripheral target organs or peripheral nerves (Heumann et al., 1987; Lindholm et al., 1987), displays no such repressive activity on GAP-43 expression by adult DRG neurons in vitro.

NGF appears to up-regulate GAP-43 expression in the neuron-like PC-12 cell line (Federoff et al., 1988), but this may simply represent conversion of PC-12 cells from a non-neuronal to a neuronal phenotype. One previous study has reported an increase in GAP-43 mRNA in cultures of adult rat DRG neurons chronically exposed to NGF (Mohiuddin et al., 1995), while another previous study reported no increase (Hu-Tsai et al., 1994). Any lack of correspondence between changes in GAP-43 mRNA and changes in GAP-43 protein may indicate that regulation of GAP-43 protein abundance is governed in part by post-transcriptional mechanisms (Perrone-Bizzozero et al., 1993; Stewart et al., 1995). Our finding of a small, but significant increase in the per neuron content of GAP-43 in cultures treated with anti-NGF may reflect the selective loss of a subpopulation of neurons which contain less GAP-43.

In a previous study, a 'priming' effect of prior peripheral axotomy on GAP-43 mRNA abundance was seen in cultured adult DRG neurons (Hu-Tsai et al., 1994). We observed a similar priming effect on GAP-43 protein abundance, persisting at least until 7 days in vitro. It should be noted that dissection and dissociation of the DRG neurons involves loss of virtually their entire axonal arbor, and therefore loss of all prior existing GAP-43 protein not located in the cell body. Thus it would appear that the priming effect of prior peripheral lesion involves enhanced production of GAP-43 protein in vitro. This might be accomplished through further upregulation of the GAP-43 synthetic apparatus in 'primed', cultured neurons. However, time course analysis of the 'priming' phenomenon in our cultures (data not shown) suggests an alternative explanation, that increased GAP-43 abundance at 7 days results from elimination of a delay in initiating elevated GAP-43 synthesis, which normally occurs at the beginning of the culture period.

GAP-43 abundance in standard, or lesion-primed, cultures was dramatically reduced by dbcAMP, a cell-permeant analogue of cyclic AMP. It has previously been reported that cyclic AMP reduces GAP-43 expression in Schwann cells (Scherer et al., 1994; Stewart et al., 1995). However, the rat GAP-43 gene lacks a cyclic AMP response element (CRE) (Nedevi et al., 1992). Cyclic AMP repression of GAP-43 expression likely occurs through an indirect effect on gene transcription, but may also or alternatively involve a post-transcriptional mechanism.

5. Summary

A sensitive method for studying GAP-43 protein abundance in microcultures of adult DRG neurons has been used to study the factors which can control GAP-43 expression. Since this approach is efficient and highly replicate it should provide a means for screening a wide variety of cell–cell interactions, which could potentially influence the abundance of GAP-43 protein in DRG neurons. The method is amenable to adaptation, either for studying GAP-43 in other cell types, or for analysing antigens other than GAP-43, when appropriately sensitive antibodies are available.

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References

Basi, G.S., Jacobson, R.D., Virág, I., Schilling, J. and Skene, J.H.P. (1987) Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth, Cell, 49: 785–791.

Bisby, M.A. (1982) Prolonged alteration in composition of fast transported protein in axons prevented from regenerating after injury, J. Neurobiol., 13: 377–381.

Brockes, J.P., Fields, K.L. and Raff, M.C. (1979) Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve, Brain Res., 165: 105–118.

Curtis, R., Stewart, H.J.S., Hall, S.M., Wilkin, G.P., Mirsky, R. and Jessen, K.R. (1992) GAP-43 is expressed by nonmyelin-forming Schwann cells of the peripheral nervous system, J. Cell Biol., 116: 1455–1464.

Dani, J.W., Armstrong, D.M. and Benowitz, L.I. (1991) Mapping the development of the rat brain by GAP-43 immunocytochemistry, Neuroscience, 40: 277–287.

De la Monte, S.M., Federoff, H.J., Ng, S.-C., Grabczyk, E. and Fishman M.C. (1989) GAP-43 gene expression during development: persistence in a distinctive set of neurons in the mature central nervous system, Dev. Brain Res., 46: 161–168.

Federoff, H.J., Grabczyck, E. and Fishman, M.C. (1988) Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids, J. Biol. Chem., 263: 19 290–19 295.

Goslin, K., Schreyer, D.J., Skene J.H.P. and Banker G. (1988) Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones, Nature, 336: 672–674.

Goslin, K., Schreyer, D.J., Skene, J.H.P. and Banker, G. (1990) Changes in the distribution of GAP-43 during the development of neuronal polarity, J. Neurosci., 10: 588–602.

Heumann, R., Korsching, S., Bandtlow, C. and Thoenen, H. (1987) Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection, J. Cell Biol., 104: 1623–1631.

Hu-Tsai, M., Winter, J., Emson, P.C. and Woolf, C.J. (1994) Neurite outrgowth and GAP-43 mRNA expression in cultured adult rat dorsal root ganglion neurons: effects of NGF or prior peripheral axotomy, J. Neurosci. Res., 39: 634–645.

Jacobsen, R.D., Virág, I. and Skene, J.H.P. (1986) A protein associated with axon growth, GAP-43, is widely distributed and developmentally regulated in rat CNS, J. Neurosci., 6: 1843–1855.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227: 680–685.

Lindholm, D., Heumann, R., Meyer, M. and Thoenen, H. (1987) Interleukin-1 regulates nerve growth factor (NGF) synthesis in the non-neuronal cells of the rat sciatic nerve, Nature, 330: 658–659.

Lindsay, R.M. (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons, J. Neurosci., 8: 2394–2405.

Lowry, O.H., Rosebrough, N.J., Farr, L. and Randall, B.J. (1951) Protein measurement with the Folin phenyl reagent, J. Biol. Chem., 193: 265–275.

McGuire, C.B., Snipes, G.J. and Norden, J.J. (1988) Light-microscopic immunolocalization of the growth-and plasticity-associated protein GAP-43 in the developing rat brain, Dev. Brain Res., 41: 277–291.

Mohiuddin, L., Fernandez, K., Tomlinson, D.R. and Fernyhough, P. (1995) Nerve growth factor and neurotrophin-3 enhance neurite outgrowth and up-regulate the levels of messenger RNA for growth-associated protein GAP-43 and Tα1 α-tubulin in cultured adult rat sensory neurones, Neurosci. Lett., 185: 20–23.

Nedevi, E., Basi, G.S., Akey, I.V. and Skene, J.H.P. (1992) A neuronal-specific GAP-43 core promoter located between unusual DNA elements that interact to regulate its activity, J. Neurosci., 12: 691–704.

Perrone-Bizzozero, N.I., Cansino, V.V. and Kohn, D.T. (1993) Post-transcriptional regulation of GAP-43 gene expression in PC12 cells through protein kinase C-dependent stabilization of the mRNA, J. Cell Biol., 120: 1263–1270.

Radola, B.J. (1984) High-resolution preparative isoelectric focusing, Methods Enzymol., 104: 256–275.

Reynolds, M.L., Fitzgerald, M. and Benowitz, L.I. (1991) GAP-43 expression in developing cutaneous and muscle nerves in the rat hindlimb, Neuroscience, 41: 201–211.

Scherer, S.S., Xu, Y.-T., Roling, D., Wrabetz, L., Feltri, M.L. and Kamholz, J. (1994) Expression of growth associated protein-43 kD in Schwann cells is regulated by axon-schwann cell interactions and cAMP, J. Neurosci. Res., 38: 575–598.

Schreyer, D.J. and Skene, J.H.P. (1991) Fate of GAP-43 in ascending spinal axons of DRG neurons after peripheral nerve injury: delayed accumulation and correlation with regenerative potential, J. Neurosci., 11: 3738–3751.

Schreyer, D.J. and Skene, J.H.P. (1993) Injury-induced GAP-43 expression displays axon branch specificity in adult rat dorsal root ganglion neurons, J. Neurobiol., 24: 959–970.

Sedgwick, J.D. and Czerkinsky, C. (1992) Detection of cell-surface molecules, secreted products of single cells and cellular proliferation by enzyme immunoassay, J. Immunol. Methods, 150: 159–175.

Skene, J.H.P. (1989) Axonal growth-associated proteins, Annu. Rev. Neurosci., 12: 127–156.

Stewart, H.J.S., Cowen, T., Curtis, R., Wilkin, G.P., Mirsky, R. and Jessen, K.R. (1992) GAP-43 immunoreactivity is widespread in the autonomic neurons and sensory neurons of the rat, Neuroscience, 47: 673–684.

Stewart, H.J.S., Curtis, R., Jessen, K.R. and Mirsky, R. (1995) TGF-βs and cAMP regulate GAP-43 expression in Schwann cells and reveal the association of this protein with the *trans*-Golgi network, Eur. J. Neurosci., 7: 1761–1772.

Tetzlaff, W., Zwiers, H., Lederis, K., Cassar, L. and Bisby, M.A. (1989) Axonal transport and localization of B-50/GAP-43-like immunoreactivity in regenerating sciatic and facial nerves of the rat, J. Neurosci., 9: 1303–1313.

Verge, V.M.K., Tetzlaff, W., Richardson, P.M. and Bisby, M.A. (1990) Correlation between GAP43 and nerve growth factor receptors in rat sensory neurons, J. Neurosci., 10: 926–934.

Young, L.M., Harrington, K.C., Tetzlaff, W. and Bisby, M.A. (1992) GAP-43 in degenerating peripheral nerve: axonal and Schwann cell origin, Soc. Neurosci. Abstr., 18: 605.