Stromelysin Generates a Fibronectin Fragment that Inhibits Schwann Cell Proliferation

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Abstract. Our previous report (Muir, D., S. Varon, and M. Manthorpe. 1990. *J. Cell Biol.* 109:2663–2672) described the isolation and partial characterization of a 55-kD antiproliferative protein found in Schwann cell (SC) and schwannoma cell line–conditioned media and we concluded that SC proliferation is under negative autocrine control. In the present study the 55-kD protein was found to possess metalloprotease activity and stromelysin immunoreactivity. The SC-derived metalloprotease shares many properties with stromelysin isolated from other sources including the ability to cleave fibronectin (FN). Furthermore, limited proteolysis of FN by the SC-derived protease generated a FN fragment which itself expresses a potent antiproliferative activity for SCs. The active FN fragment corresponds to the 29-kD amino-terminal region of the FN molecule which was also identified as an active component in SC CM. Additional evidence that a proteolytic fragment of FN can possess antiproliferative activity for SCs was provided by the finding that plasmin can generate an amino-terminal FN fragment which mimicked the activity of the SC metalloprotease-generated antiproliferative FN fragment. Both the 55-kD SC metalloprotease and the 29-kD FN fragment could completely and reversibly inhibit proliferation of SCs treated with various mitogens and both were largely ineffective at inhibiting proliferation by immortalized or transformed SC lines. Normal and transformed SC types do secrete the proform of stromelysin, however, transformed cultures do not produce activated stromelysin and thus cannot generate the antiproliferative fragment of FN. These results suggest that, once activated, a SC-derived protease similar to stromelysin cleaves FN and generates an antiproliferative activity which can maintain normal SC quiescence in vitro.

SCHWANN cell (SC)[1] proliferation is highly regulated and essentially occurs only during development and regeneration. During development, SCs recognize and adhere to axons and then are stimulated to proliferate and eventually populate the entire length of the axon (Webster and Favilla, 1984). In healthy adult peripheral nerve, SCs generally do not proliferate but they apparently can reenter the mitotic cycle during Wallerian degeneration induced by nerve trauma. These same SC behaviors can be reproduced in vitro in that cultured SCs contact and adhere to neuronal processes and then proliferate in response to a neuritic mitogenic signal (Salzer and Bunge, 1980). As in mature nerve, SCs in established co-cultures eventually cease to proliferate even in the continued presence of potentially mitogenic neurons. Isolated SCs divide infrequently under standard culture conditions and we have demonstrated that SCs release a factor(s) into their culture medium which inhibits proliferation in an autocrine manner (Muir et al., 1990*a*). From conditioned medium (CM) a 55-kD protein was isolated and partially characterized that completely and reversibly inhibits proliferation by SCs treated with a variety of mitogens including that presented by regenerating neurons in co-culture. Several molecular forms of antiproliferative activity were found in SC CM. The 55-kD "Neural Antiproliferative Protein," or NAP, exists in a free form and can be separated from a high molecular mass complex. In addition, a lower mass form (≈30 kD) of antiproliferative activity was found in SC CM.

Several characteristics of the antiproliferative activities and specifically those of the 55-kD NAP suggested the possibility that proteolytic activation might be involved in the inhibition of SC proliferation. The present study tested the hypothesis that a SC-derived protease can cleave and activate a substrate in the culture medium which possesses a cryptic antiproliferative activity for SCs. Results showed the 55-kD NAP is immunologically related to and shares metalloprotease activity with stromelysin. We now can attribute the previously described SC CM–derived ≈30-kD form of antiproliferative activity to a 29-kD amino-terminal, heparin-

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1. *Abbreviations used in this paper*: APMA, 4-aminophenylmercuric acetate; APU, antiproliferative units; BrdU, bromodeoxyuridine; CAFF, cryptic antiproliferative fibronectin fragment; CM, conditioned medium; NAP, neural antiproliferative protein; NEM, *N*-ethylmaleimide; SC, Schwann cell.

binding FN fragment proteolytically generated by the SC-derived metalloprotease. Preparations of this "Cryptic Antiproliferative Fibronectin Fragment", or CAFF, were obtained by incubating the 55-kD NAP with FN purified from the plasma of several species. A plasmin-generated FN fragment, with similar molecular characteristics to CAFF and which was previously reported to inhibit the proliferation of endothelial cells (Homandberg et al., 1985, 1986), was also generated and it too expressed antiproliferative activity for SCs.

Materials and Methods

Cell Culture

Purified populations of quiescent SCs were obtained from dissociated neonatal rat sciatic nerves as previously described (Muir et al., 1989*a*). Loss of normal growth control (immortalization) of secondary SCs was achieved by continuous treatment for 100 d with 20 ng/ml cholera toxin (Sigma Chemical Co., St. Louis, MO) as described (Muir et al., 1990*a*). RN22 and D6P2T Schwannoma cells (Bansal and Pfeiffer, 1987; Pfeiffer and Wechsler, 1972) were cultured in DME containing 10% calf serum. Conditioned media were collected from dense (3–5 × 10^6 cells/75 cm^2) cultures of SCs (SC CM) and RN22 Schwannoma (RN22 CM), thoroughly washed with HBSS, and then incubated in serum-free DME for 24–48 h.

Schwann Cell Proliferation Assay and Immunostaining for Bromodeoxyuridine

Quiescent SC microcultures were established in polyornithine-treated 96-well plates containing 14,000 cells/6-mm well in 100 μl of DME + 10% calf serum as described (Muir et al., 1990*b*). For some assays, SCs were seeded in polyornithine-coated wells treated with 50 μl of a 2 μg/ml solution of rat L2 yolk sac tumor laminin (prepared as described by Engvall et al., 1983). SC/neuron co-cultures were established by adding 14,000 SCs and 2,000 embryonic day 8 ciliary ganglionic motor neurons to each microwell as described (Muir et al., 1989*a*). For routine assays of antiproliferative activity on mitogen-stimulated SCs, microcultures were seeded in DME + 10% calf serum containing 20 ng/ml cholera toxin. Serially diluted test samples were presented for 72 h and bromodeoxyuridine (BrdU) (Sigma Chemical Co.) was added to a concentration of 10 μM during the final 24 h. SC proliferation was assessed by direct cell counting and BrdU incorporation into DNA was measured by an ELISA performed on fixed monolayer microcultures as previously described (Muir et al., 1990*b*). Briefly, following BrdU incorporation, the microcultures were fixed by 70% ethanol and the DNA denatured by incubation with 2 M HCl for 10 min at 37°C. BrdU-DNA was labeled using monoclonal anti-BrdU antibody (Dako-Patts Corp., Santa Barbara, CA) (50 μl/well; 1 μg/ml) and bound antibody was detected by peroxidase-conjugated rabbit anti-mouse IgG (Dako-Patts Corp.) (50 μl/well; 2 μg/ml). The colorimetric substrates *o*-phenylenediamine (0.05%) and H_2O_2 (0.02%) were added in 80 μl of 50 mM phosphate/citrate buffer at pH 5 and the reaction was terminated after 5–20 min by adding 40 μl of 2 M sulfuric acid. The adsorbance was measured at 490 nm by a microplate reader (MR600; Dynatech Labs, Alexandria, VA) interfaced with a computer. Using cholera toxin-stimulated SCs, the titer of each sample in antiproliferative units (APU)/ml was expressed as the sample dilution required to inhibit by 50% the maximal incorporation of BrdU into DNA (BrdU-DNA immunoactivity). The percentage of cells with BrdU-DNA was determined by immunostaining as described above in the ELISA except the insoluble chromagen diaminobenzedine-tetrahydrochloride was used. Proliferation assays using immortalized SC and rat schwannoma cell lines were performed as described above for SCs except that no mitogens were added to the serum-supplemented medium.

NAP Isolation

An effective but low-yield purification scheme was used to isolate the 55-kD NAP by dissociation from a high molecular weight complex followed by preparative gel electrophoresis as previously described (Muir et al., 1990*a*). Alternatively, a NAP-enriched fraction was obtained by the following fractionation sequence and a step-by-step monitoring of antiproliferative activity using the BrdU-ELISA of mitogen-stimulated SCs. Serum-free SC or RN22 CM (1 liter) adjusted to pH 7.8 and passed over a DEAE column (2 × 10 cm) resulted in a twofold increase in the total NAP activity in the nonbinding fraction (probably because of the removal of a DEAE-binding NAP inhibitor). The NAP sample was then applied to heparin-sepharose and the active nonbinding fraction was concentrated 200-fold and equilibrated with PBS by ultrafiltration using a 10-kD cut-off filter (Amicon Corp., Danvers, MA). The concentrate was submitted to CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration performed on a 2.5 × 100 cm column equilibrated with PBS and the antiproliferative activity eluting in one major peak with a molecular mass corresponding to ≈55 kD was collected and concentrated. Heparin-affinity chromatography was performed on a 1.4 × 7 cm column equilibrated with 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M NaCl. A heparin-binding fraction was eluted in one step with 0.5 M NaCl in phosphate buffer. The preparation from serum-free RN22 CM contained ≈50 μg total protein and 1,600 APU. The sample contained ≈8 μg of NAP and thus was ≈15% NAP protein and was used for many preliminary studies later to be repeated with the electrophoretically homogeneous preparation described previously.

Immunoassays for Stromelysin

Fractions generated during isolation of the 55-kD NAP were examined for stromelysin by enzyme-linked immunosorbent assay as described (Engvall, 1980). The anti-stromelysin mAbs used recognize both prostromelysin and activated stromelysin and were a generous gift from Dr. Scott Wilhelm (Miles Laboratories, West Haven, CT). The NAP-enriched preparations were examined by Western immunoblotting as previously described (Muir et al., 1989*b*).

Immunosequestration of stromelysin from NAP-enriched preparations was performed to test if antiproliferative activity resided in this antigen. The anti-stromelysin mAbs were mixed with protein A-Sepharose (Pharmacia Fine Chemicals) in 0.5 M glycine buffer (pH 8.9) containing 3 M NaCl and incubated for 1 h. The sepharose was collected by brief centrifugation, washed, and then mixed with NAP-enriched preparations. After a 2-h incubation, the material bound to the immuno-sepharose was removed by centrifugation and the resulting supernatants were assayed for NAP activity and residual stromelysin immunoreactivity by ELISA. Similar immunosequestration methods using protein A-Sepharose and mouse polyclonal antibodies raised against the antiproliferative amino-terminal FN fragment were used to identify the active component in the 30-kD fraction of SC CM.

Zymography

Substrates were cross-linked into the acrylamide/bis-acrylamide resolving gels by adding 1 mg/ml solubilized alpha-casein. Samples were electrophoresed under nonreducing conditions at 4°C according to Laemmli (1970). The gels were washed 3 × 10 min with 2.5% Triton X-100 to remove SDS and then washed with 50 mM Tris-HCl, pH 8.0. Protease digestion of the cross-linked substrate progressed during incubation of the gel for 16–32 h at 37°C in the same buffer containing 5 mM $CaCl_2$. As specified, the gels were incubated in buffer containing the protease inhibitors 1, 10-phenanthroline (10 mM), EDTA (10 mM), PMSF (0.5 mM), *N*-ethylmaleimide (NEM) (10 mM), aprotinin (1 μg/ml), cystatin (1 μg/ml), or pepstatin (1 μg/ml). Following incubations, the gels were fixed with 50% methanol/10% acetic acid, stained with 0.2% Coomassie blue R250 and then destained in a diluted fix solution.

Substrate Cleavage by the CM-derived 55-kD Protease

RN22 CM is an abundant source for isolating the 55-kD metalloprotease (a latent enzyme) and this preparation could be activated by treatment with 4-aminophenylmercuric acetate (APMA) or trypsin and was most active in the presence of Ca^{2+}. For analytical purposes the 55-kD metalloprotease was incubated at various enzyme/substrate ratios for 48–72 h at 37°C (30°C for type I Collagen) in 25 mM Tris-HCl, pH 8.0 containing NaCl (100 mM), $CaCl_2$ (5 mM), APMA (1 mM), PMSF (0.5 mM), NEM (10 mM), and aprotinin (1 μg/ml). The cleavage products were mixed with Laemmli sample buffer with and without β-mercaptoethanol (5%) and analyzed on 5–15% acrylamide gels and then stained with Coomassie blue R250 in 50% methanol and 10% acetic acid. The substrates tested were FN (prepared as described below), laminin (Bethesda Research Laboratories, Gaithersburg, MD), native type I collagen (Vitrogen Collagen Corp.), and native IV collagen (Collaborative Research Inc., Bedford, MA).

Fibronectin Degradation and Isolation of the 29-kD CAFF

Bovine and rat plasma FNs were isolated from citrated plasma (Pel Freez Biologicals, Rogers, AR) by two cycles of gelatin–affinity chromatography (Engvall and Ruoslahti, 1977) using 4 M urea to elute bound FN. The eluted fraction was concentrated by ultrafiltration using a 100-kD cut-off filter (Amicon Corp.) and the concentrate was submitted to CL4B gel filtration and the peak fractions of ≈400–440 kD were collected. Ferritin (440 kD) was used as a molecular mass standard for calibration. Unfragmented human plasma FN was prepared by submitting human plasma FN (New York City Blood Center, New York, NY) to CL4B gel filtration.

To analyze the proteolytic fragments, 1 mg of purified FN was mixed with ≈8 μg of the 55-kD metalloprotease preparation and incubated for 72 h at 37°C in 25 mM Tris-HCl, pH 7.6 containing NaCl (100 mM), $CaCl_2$ (5 mM), APMA (1 mM), PMSF (0.5 mM), NEM (10 mM), and aprotinin (1 μg/ml). For preparative yields of FN fragments, 25 mg of FN was digested with 500 μg of porcine plasmin (Sigma Chemical Co.) for 20 h at 37°C in 25 mM Tris-HCl, pH 8.4 containing NaCl (100 mM) and lysine (10 mM). Each digest was applied to the following sequence of 10 ml columns (1.4 × 7 cm) connected in tandem: DEAE-cellulose (Whatman Laboratory Products Inc., Clifton, NJ), gelatin-sepharose (Pharmacia Fine Chemicals), and heparin–sepharose (Pharmacia Fine Chemicals). The columns were uncoupled and the bound materials were eluted from DEAE with 0.5 M NaCl, from gelatin with 4 M urea, and from heparin with 0.5 M NaCl. The elutes were concentrated by PM10 ultrafiltration (Centriprep; Amicon Corp.) and equilibrated with PBS. The heparin-binding, 0.5 M NaCl eluted concentrate was found to be greatly enriched in antiproliferative activity and was further fractionated by S200 (superfine; Pharmacia Fine Chemicals) gel filtration in PBS. All fractionations were performed at 4°C. The fractions were assayed for antiproliferative activity using SC microcultures as described above and most were also analyzed on SDS gels. The heparin-binding, antiproliferative activity eluting from gel filtration corresponding to 29 kD was tested for an ability to bind actin using a modification of the methods described by Keski-Oja and co-workers (1980). Amino acid analysis was performed on a Sequenator (Applied Biosystems, Foster City, CA).

Results

NAP Copurifies with a Metalloprotease Activity

We previously reported (Muir et al., 1990*a*) that media conditioned by rat SC cultures and by schwannoma cell lines contained an identical antiproliferative activity which can completely inhibit proliferation by mitogen-stimulated SCs. Gel filtration of either CM resolved three distinct forms of antiproliferative activity and a predominant 55-kD component, termed NAP, was isolated and partially characterized. NAP isolated from CM exhibited several properties which suggested it might contain proteolytic activity. For instance, by removing the DEAE-binding material from CM, the total antiproliferative activity collected in the nonbinding fraction was increased, suggesting an inhibitor was removed. In addition, concentration of the CM or activity-enriched fractions caused further increases, perhaps resulting from activation by proteases. This possibility was supported by the finding that the antiproliferative activity of the 55-kD NAP was increased by mild treatment with trypsin. In addition, inactivation resulted from treatments with heat and disulfide-reducing agents. Each of these observations is also consistent with the speculation that the 55-kD NAP itself possesses proteolytic activity. To test this idea, an electrophoretically pure preparation of NAP (Fig. 1 *A*, lane *1*) was analyzed by substrate–overlay gel electrophoresis (zymography). The results are shown in Fig. 1 *A*. A single zymographic band with caseinolytic activity appeared with a relative molecular mass of 55 kD (lane *2*). This proteolytic profile was eliminated when zymography was performed in the presence of the metal chelator 1, 10-phenanthroline (lane *3*), indicating the NAP preparation contained a 55-kD metalloprotease. Other protease inhibitors (i.e., PMSF, NEM, aprotinin, cystatin) did not diminish the protease band (results not shown).

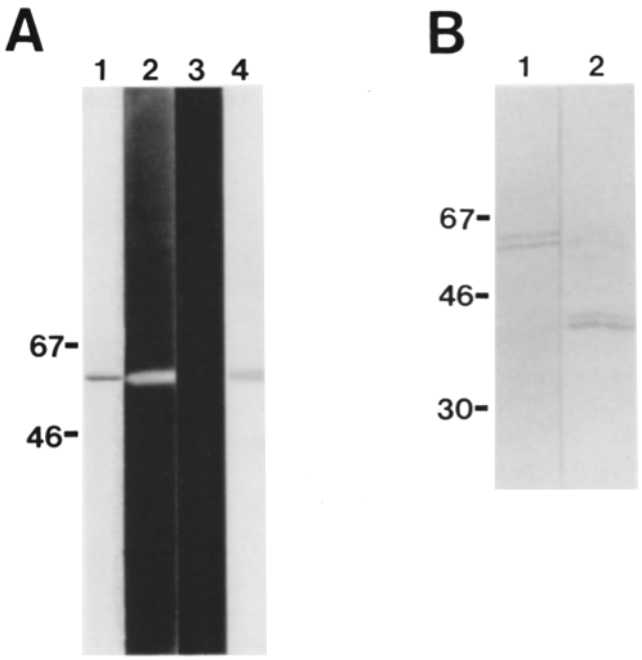

Figure 1. Casein-degrading activity and anti-stromelysin immunoreactivity associated with the 55-kD NAP. (*A*) A highly enriched preparation of the 55-kD NAP obtained from serum-free RN22 CM was examined by nonreducing SDS-PAGE and protein staining (lane *1*). On zymographic gels the 55-kD NAP contained a 55-kD caseinolytic activity (lane *2*) which was eliminated when zymography was performed in the presence of the zinc chelator 1, 10-phenanthroline (lane *3*). The RN22 CM–derived NAP was examined for anti-stromelysin immunoreactivity by Western immunoblotting (lane *4*). Molecular weight designations represent the migration positions of BSA (67 kD) and ovalbumin (46 kD) on 12% acrylamide standard and zymographic gels and on Western blots. (*B*) Crude serum-free CM was collected after 2 d from dense cultures of RN22 Schwannoma (lane *1*) and isolated SCs (lane *2*) and then concentrated 300-fold by ultrafiltration (10-kD cut off). The concentrate (20 μl) was run on 12% acrylamide mini-gels under nonreducing conditions, electroblotted to nitrocellulose, and immunoperoxidase stained using monoclonal anti-stromelysin antibodies as described in Materials and Methods. Molecular weight designations represent the migration positions of BSA (67 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD).

NAP Copurifies with Stromelysin Immunoreactivity

The properties of the SC-derived 55-kD metalloprotease closely paralleled those reported by Chin and co-workers (1985) for stromelysin and the rat equivalent, transin (Umenishi et al., 1990). Consequently, when fractions were assayed for stromelysin immunoreactivity by ELISA, 55-kD NAP activity and stromelysin immunoreactivity were found to co-purify in each of our purification steps. Furthermore, the 55-kD metalloprotease activity demonstrated by zymography was shown by Western immunoblotting to co-migrate

with a band stained by mAbs to stromelysin (Fig. 1 *A*, lane *4*). To determine the forms of stromelysin immunoreactivity present in CMs, serum-free SC CM and RN22 CM were concentrated and then examined by Western immunoblotting. Immunostaining was performed using a cocktail of specific mAbs which recognize prostromelysin and activated stromelysin (Wilhelm et al., 1991). Both SC and RN22 CMs contained 55–58 kD (postromelysin) and 41–44 kD (activated stromelysin) forms, each appearing as band pairs. The heterogeneity within each form is reportedly attributable to partial glycosylations (Wilhelm et al., 1987). Interestingly, while RN22 CM and SC CM contained both forms, RN22 CM contained predominantly prostromelysin (and only a trace of the lower mass form) while in SC CM the activated form was much more abundant. These results, shown in Fig. 1 *B*, lanes *1* and *2*, suggest that SC cultures are capable of converting prostromelysin to an active form whereas the transformed RN22 cultures are not.

Attempts to determine the contribution of stromelysin to the antiproliferative activity in CM samples were complicated by the presence of more than one form of activity. Immunosequestration using anti-stromelysin antibodies bound to protein A-Sepharose was only marginally effective at reducing the total activity present in crude CM. Previous results have shown three distinct forms of antiproliferative activity in the CMs but immunosequestration performed on enriched fractions of the high molecular mass (>1,000 kD) and lower mass (≈30 kD) forms were ineffective at reducing antiproliferative activities. However, for enriched preparations of 55-kD NAP >60% of the antiproliferative activity was removed by a single cycle immunosequestration. Additional cycles depleted more activity. Thus, it appeared likely that the 1,000- and 30-kD forms were not immunologically the same as the 55-kD NAP, but that the 55-kD antiproliferative factor is immunologically similar to stromelysin.

Substrate Specificity for the SC-derived Metalloprotease

Next, the 55-kD metalloprotease was tested for an ability to degrade various extracellular matrix proteins including FN, laminin, and native types I and IV collagens. After incubation with the 55-kD metalloprotease (and APMA), only FN appeared significantly degraded when the proteolytic samples were examined by SDS–gel electrophoresis performed under nonreducing conditions. The results of FN degradation are shown in Fig. 2 (lane *2*). The large proteolytic fragments had relative molecular masses of 140–160 kD and a major cleavage product appeared with a mass of 29 kD. FN degradation was attributed solely to metalloprotease activity in that it was selectively abolished when digestion was attempted in the presence of 1, 10-phenanthroline (lane *1*). These results indicated that, of the extracellular matrix proteins tested, only FN was cleaved into distinct fragments. However, further examination of the 55-kD protease–treated samples on gels run under reducing conditions showed laminin and type IV collagen could be partially proteolysed while type I collagen apparently was unaffected by the metalloprotease activity (results not shown). The substrate tests for the SC-derived, 55-kD metalloprotease gave results consistent with those reported for rabbit fibroblast stromelysin (Chin et al., 1984).

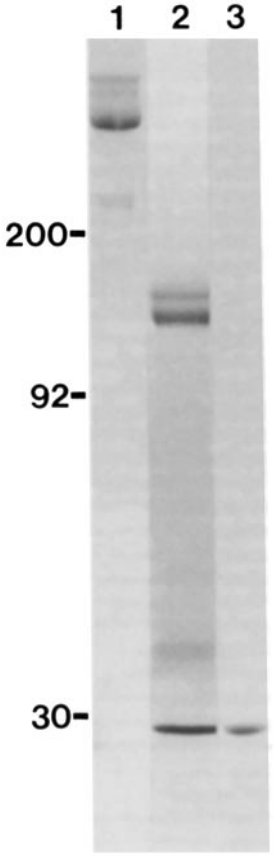

Figure 2. Proteolytic FN fragments generated by the SC-derived 55-kD metalloprotease. Bovine plasma FN (1 mg) was mixed with ≈8 μg of the 55-kD metalloprotease and incubated for 72 h at 37°C in 25 mM Tris-HCl, pH 7.6, containing NaCl (100 mM), $CaCl_2$ (5 mM), APMA (1 mM), PMSF (0.5 mM), NEM (10 mM), and aprotinin (1 μg/ml). Incubation was performed in the presence (lane *1*) or absence (lane *2*) of the metalloprotease inhibitor 1, 10-phenanthroline. The samples were electrophoresed on 5–15% acrylamide gradient gels under nonreducing conditions and then stained with Coomassie blue. The digest shown in lane *2* was applied to tandem columns of DEAE–, gelatin–, and heparin–sepharose. The fragments which did not bind to DEAE or gelatin but did bind to heparin were eluted from the heparin column by 0.5 M NaCl. The heparin-binding, 0.5 M NaCl-eluted fraction was concentrated by ultrafiltration (10-kD cut off) and was further fractionated by S200 (superfine) gel filtration in PBS. The fractions corresponding to ≈30 kD were pooled and examined by SDS-PAGE (lane *3*) as described above. The migration positions of myosin (200 kD), phosphorylase b (92 kD), and carbonic anhydrase (30 kD) are indicated.

Proteolysis of FN Generates a Cryptic Antiproliferative Activity

The proteolytic mixture of FN and 55-kD protease was then fractionated by gel filtration and the resulting eluates were assayed for antiproliferative activity. The results are shown in Fig. 3. Interestingly, antiproliferative activity was found in two distinct peaks; in addition to the 55-kD peak corresponding to the added NAP (55 kD, prostromelysin) a peak of new activity appeared in the elution profile at ≈30 kD, which was enriched in a 29-kD FN fragment demonstrated in Fig. 2. If the 55-kD metalloprotease was converted (by APMA) to an active form (M_r = 41–44 kD) during the proteolytic incubations, this form was apparently labile or did not express antiproliferative activity under the conditions of the SC assay. The antiproliferative, 30-kD gel filtration fraction did not show zymographic activity and was characterized further by affinity-chromatography steps aimed at isolating fragments of FN with known binding properties. The results are shown in Table I. First, the antiproliferative activity did not bind anion-exchange resin (at pH 8.0) or gelatin–sepharose. The activity-enriched, nonbinding fraction was then applied to heparin–sepharose, which bound most of the activity. The heparin-binding antiproliferative activity was eluted by 0.5 M NaCl and appeared on SDS gels as a nearly homogeneous band of 29 kD (Fig. 2, lane *3*). The 29-kD protein was resistant to dansylation and Edman degrada-

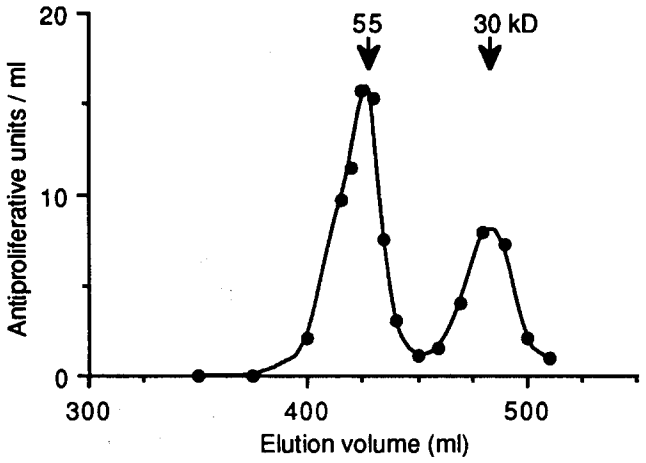

Figure 3. Antiproliferative activity in gel filtration fractions of a proteolytic mixture of the 55-kD protease and FN. Bovine FN was degraded by incubation with the 55-kD protease as described for Fig. 2, lane *2*. The proteolytic mixture was concentrated and fractionated by gel filtration and the eluted fractions were assayed for antiproliferative activity using cholera toxin–stimulated SCs as described in Materials and Methods. Estimated molecular masses for the two peaks of antiproliferative activities are indicated by arrows. Data represent the means of duplicate determinations from two separate experiments.

tion (Table I), indicating that it had a modified (blocked) amino terminus. Taken together, the properties of the 29-kD fraction indicated that we had generated and isolated a 29-kD, amino-terminal FN fragment (CAFF) with potent antiproliferative activity for SCs. The cryptic 29-kD activity was potent with an ED_{50} = 60 nM and DNA synthesis by mitogen-stimulated SCs could be completely inhibited by 10 μg/ml (see Fig. 7 *D*). Nearly identical results were obtained when either bovine (shown in lane *3*), human, or rat FN (results not shown) were treated with the 55-kD protease and the digests fractionated as described above. We had previously observed that heparin does not bind the 55-kD NAP, thus heparin-affinity can be used to separate the proteolytic (NAP) and product (CAFF) forms of antiproliferative activity.

In a previous report (Muir et al., 1990*a*) the antiproliferative activity profile obtained by gel filtration of crude, serum-containing SC CM also contained a peak at ≈30 kD. To determine whether this form of activity might be attributed to a fragment of FN (generated from serum FN) the 30-kD SC CM-derived antiproliferative fraction (see Fig. 5, Muir et al., 1990*a*) was submitted to the same fractionation sequence used to isolate the antiproliferative bovine FN fragment described above. As shown in Table I, the 30-kD SC CM-derived activity did not bind DEAE or gelatin but did bind heparin. In addition, the heparin-binding material contained a component which appeared on SDS gels as a 29-kD protein (Fig. 4, lane *1*) which co-migrated with a band on Western immunoblots stained by anti-FN polyclonal antibodies (Fig. 4, lane *2*). In addition, >80% of the antiproliferative activity expressed by the 30-kD SC CM fraction was removed by immunosequestration methods using antibodies raised against the amino-terminal FN fragment. We conclude from these findings that SC CM contains a 29-kD antiproliferative FN fragment with properties similar to that

Table I. Antiproliferative Activity in Fractions of Proteolytic Fibronectin Fragments

Property	Antiproliferative activity in sample (attributable to the FN fragment)*		
	55-kD protease+FN	plasmin+FN	30-kD SC CM
DEAE binding	−	−	−
Gelatin binding	−	−	−
Heparin binding	+	+	+
Actin binding	+	+	n.d.
N blocked	+	+	n.d.
Molecular mass	29 kD	29 kD	29 kD

Three preparations containing FN fragments were found to express potent antiproliferative activity for SCs. The proteolytic samples *55-kD protease +FN*, *plasmin + FN*, and *30-kD SC CM* were prepared as described in Materials and Methods. Each sample contained proteolytic FN fragments and newly generated antiproliferative activity. The samples were adjusted to pH 8.0 and then were applied to columns of DEAE–, gelatin–, heparin–, and actin–sepharose. For each sample the heparin-binding antiproliferative fraction was further purified by S200 gel filtration and examined by SDS-PAGE. All fractions were assayed for antiproliferative activity using cholera toxin-stimulated SCs (scored as expressing + or not expressing − activity).

* The proteolytic samples inherently contained antiproliferative constituents with known properties; the APMA-activated, 55-kD protease does not bind DEAE, gelatin, heparin, or actin and plasmin is not antiproliferative within the concentration range used here.

generated by experimental proteolysis of FN by the purified SC-derived protease.

A Plasmin-generated FN Fragment with Antiproliferative Activity

Our findings indicate that a SC protease with similarities to stromelysin can degrade FN and generate a 29-kD heparin–binding fragment which expresses antiproliferative activity. Efforts to obtain greater amounts of this proteolytic fragment for detailed characterization were hampered by the scarcity of purified SC-derived protease. However, we considered the possibility that we had isolated an antiproliferative FN fragment with molecular properties similar to those reported for the 29-kD amino-terminal fragment of human FN (McDonagh et al., 1981; Homandberg et al., 1985).

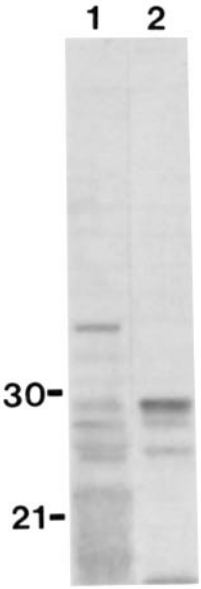

Figure 4. FN immunoreactivity in a heparin-binding, 30-kD fraction obtained from serum-supplemented SC CM. Serum-supplemented medium conditioned for 3 d by quiescent SC cultures was applied to tandem columns of DEAE–, gelatin–, and heparin–sepharose. The material which did not bind to DEAE or gelatin but did bind to heparin was eluted from the heparin column by 0.5 M NaCl and then was submitted to gel filtration. The fractions corresponding to ≈30 kD were pooled and examined by SDS-PAGE and protein staining (lane *1*) and Western immunoblotting using polyclonal anti-FN antibodies (lane *2*). The migration positions of carbonic anhydrase (30 kD) and soybean trypsin inhibitor (21 kD) are indicated.

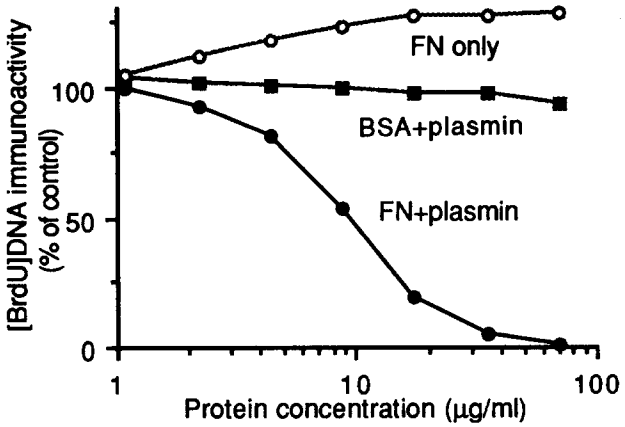

Figure 5. Proteolysis of FN by plasmin generates a cryptic antiproliferative activity. Bovine FN and BSA were degraded with plasmin as described in Materials and Methods and the proteolytic mixtures were tested for antiproliferative activity using cholera toxin–stimulated SCs. Intact FN was additive with the mitogenic effects of cholera toxin. The mixture of FN and plasmin, after 16 h of incubation, expressed a potent antiproliferative activity capable of completely inhibiting DNA synthesis. Plasmin, within the concentration range used to degrade FN did not inhibit SC proliferation and the proteolytic mixture of plasmin and BSA expressed no antiproliferative activity. Data represent the means of quadruplicate determinations from two separate experiments. (SD = <6%).

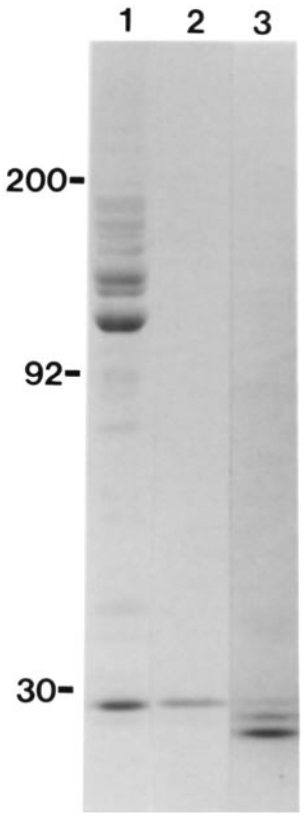

Figure 6. Proteolytic FN fragments generated by plasmin. Bovine plasma FN (25 mg) was digested with 500 μg of porcine plasmin for 20 h at 37°C in 25 mM Tris-HCl, pH 8.4 containing NaCl (100 mM) and lysine (10 mM). The digest (lane *1*) was applied to gelatin–, tandem columns of DEAE–, and heparin–sepharose. The DEAE and gelatin non-binding FN fragments that bound to heparin were eluted from the heparin column with 0.5 M NaCl. The eluted fraction was concentrated by PM10 ultrafiltration and then was submitted to S200 (superfine) gel filtration. The fraction corresponding to ≈30 kD was examined by SDS-PAGE immediately (lane *2*) or after being stored in solution for 7 d at 4°C (lane *3*). Samples were run under non-reducing conditions on 5–15% acrylamide gels which were then stained with Coomassie blue. The migration positions of myosin (200 kD), phosphorylase b (92 kD), and carbonic anhydrase (30 kD) are indicated.

Thus, plasmin was tested for an ability to generate an antiproliferative FN fragment. Using a modification of the procedures reported by McDonagh and co-workers (1981), 25 mg of bovine FN was degraded with plasmin and the proteolytic mixture tested for antiproliferative activity using cholera toxin-stimulated SCs. The results shown in Fig. 5 demonstrated that intact FN was additive with the mitogenic effects of cholera toxin while plasmin, within the concentration range used to degrade FN, did not significantly affect DNA synthesis. However, the mixture of FN and plasmin, after 16 h of incubation, expressed a potent antiproliferative activity capable of completely inhibiting DNA synthesis by SCs. To determine if the activity was attributed to an amino-terminal FN fragment, the digest (Fig. 6, lane *1*) was passed in tandem over DEAE–, gelatin–, and heparin–sepharose columns. The heparin-binding material was eluted with 0.5 M NaCl and then submitted to gel filtration. The resulting fractions were assayed for antiproliferative activity (see Table I) and a major peak of activity was collected from gel filtration corresponding to ≈30 kD. This highly enriched fraction appeared on SDS gels as a single band (or sometimes as a band pair) with 27–29 kD (Fig. 6, lane *2*). This sample was resistant to dansylation and direct Edman degradation, thus we concluded it had a blocked amino terminus and corresponded to the amino-terminal, 29-kD FN fragment described by McDonagh and co-workers (1981). From 25 mg of intact FN ~2 mg of this fragment was isolated. The specific activity of this FN fragment preparation was generally found to be between 200–400 nM (see below) although the activity was somewhat variable and labile in solution. Furthermore, the relative molecular mass on SDS gels also appeared to change with time in storage from a 29- to a 27-kD form and sometimes an apparent intermediate was seen (Fig. 6, lane *3*). It seemed that an active 29-kD form of the FN fragment would convert to a more compact configuration which greatly reduced its antiproliferative activity. The nature of this conversion is obscure and will require a more detailed examination. In comparison, the 29-kD fragment generated by the SC-derived metalloprotease was considerably more stable and retained activity for weeks in solution. The plasmin-generated fragment appeared similar to that generated by the SC-derived protease in that they both: (*a*) did not bind DEAE or gelatin; (*b*) had a moderately high affinity for heparin; (*c*) coeluted from gel filtration; (*d*) comigrated on SDS gels; and (*e*) expressed potent antiproliferative activity for SCs. Nearly identical results were obtained when either bovine (shown in Fig. 6), human, or rat FN (results not shown) were treated with plasmin and the digests fractionated as described above. These findings indicate that a plasmin-generated, 27–29-kD, heparin-binding FN fragment possessed a potent, albeit labile, antiproliferative activity for SCs.

While the isolation and characterization of the 29-kD amino-terminal FN fragment generated by plasmin are well documented, the properties of our preparation were further examined to confirm this designation for the antiproliferative activity. Additional results are shown in Table I. It is known that the heparin-binding, amino-terminal 29-kD fragment also binds actin and our preparations shared this property. In addition, no sequence information was obtained by direct Edman degradation and the fragment was resistant to dansylation. Analysis of amino acid composition gave results (not shown) consistent with published findings (McDonagh et

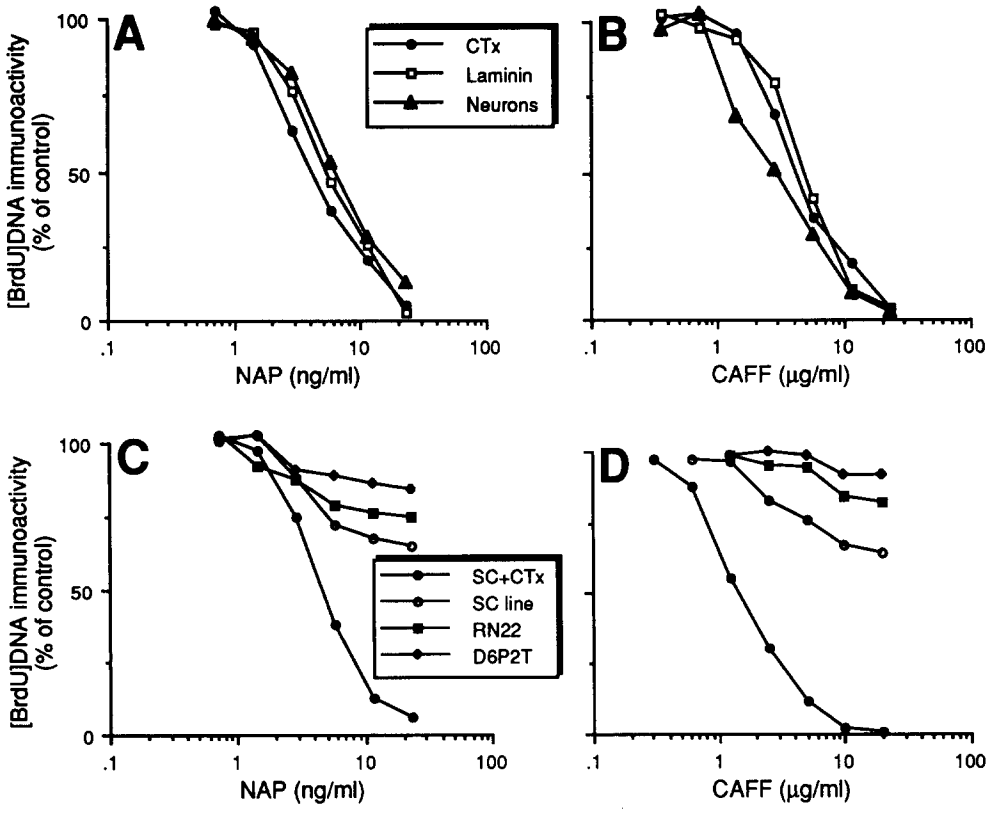

Figure 7. Comparison of NAP and CAFF activities for SC treated with various mitogens and for SC lines. Subconfluent microcultures of rat sciatic nerve SCs (14,000 cells/6-mm-diam well) were grown with 100 μl DME containing 10% calf serum with one of the following mitogens: soluble cholera toxin (20 ng/ml), polyornithine-treated wells coated with rat laminin (50 μl/well, 2 μg/ml), or co-culture with ciliary ganglionic neurons (2,000 neurons/well). These mitogen-stimulated SCs were treated for 72 h with serial dilutions of the (*A*) 55-kD NAP or (*B*) CAFF (29-kD amino-terminal FN fragment generated by the 55-kD protease). Immortalized SC and two schwannoma cell (RN22 and D6P2T lines) cultures were grown in DME containing 10% calf serum and treated with the (*C*) 55-kD NAP or (*D*) CAFF as described above. Proliferation was assessed by addition of BrdU (10 μM) to the media during the final 24 h of the 72 h treatments and immunoassays of BrdU incorporation into DNA were performed as described in Materials and Methods. Values for each condition were expressed as the percentage of maximal (BrdU)-DNA immunoactivity for cultures without NAP or CAFF treatment. Data represent the means of quadruplicate determinations from four separate experiments. (SD = <10%, except for *A*, neurons SD = <12% and *B*, neurons SD = <15%).

al., 1981; Garcia-Pardo et al., 1983). Taken together, these results indicate that a plasmin-generated, amino-terminal fragment of FN has properties very similar to the antiproliferative FN fragment generated by the SC-derived 55-kD protease and also to that found in SC CM.

Comparison of NAP and CAFF Activities

In our earlier study (Muir et al., 1990*a*) both SC CM and the 55-kD NAP inhibited proliferation by SCs stimulated with various mitogens and with co-cultured regenerating neurons. While the inhibition of SCs could be complete, proliferation by immortalized SCs and schwannoma cell lines was largely uninhibited (10–20%) by the antiproliferative activity. To test the hypothesis that NAP activity was related to the production of a proteolytic FN fragment, we first examined whether the activity of the 29-kD CAFF and the 55-kD NAP shared the above mentioned characteristics. The results are shown in Fig. 7. Similar to those obtained for NAP activity (Fig. 7 *A*), the plots of CAFF antiproliferative activity (Fig. 7 *B*) were superimposable (when expressed as percent inhibition) for SCs treated with different mitogens. In addition to finding that each activity could completely inhibit the proliferation of mitogen-stimulated SCs, both NAP (Fig. 7 *C*) and CAFF (Fig. 7 *D*) only partially inhibited immortalized SCs and schwannoma cell lines. These similarities support the idea that NAP and CAFF activities inhibit SC proliferation through the same pathway.

Discussion

Large-yield cultures of highly purified SC can be established which retain many of the characteristics of SCs in vivo, including the abilities to proliferate and differentiate in association with neurons (Porter et al., 1986). During peripheral nerve development and regeneration, SCs attach to neuronal axons and proliferate in response to a neuronal cell surface mitogen and then differentiate after retiring from the mitotic cycle. The number of SCs in differentiated nerve is generally stable even though the neuronal mitogen appears to be present since it can be extracted from axons associated with quiescent SCs (Salzer et al., 1980). Some, if not all, SCs in differentiated nerve retain an ability to proliferate in response to nerve trauma or when cultured with mitogens such as axolemmal fragments. Taken together, these observations suggest that SC homeostasis probably involves a strong inhibitory component which maintains SC quiescence in the presence of potentially mitogenic stimuli, including contact with axons.

SC proliferation appears to be under negative autocrine control. Medium conditioned by SC cultures contains potent antiproliferative activity which can account for the quiescent state of SCs even in the presence of apparent serum and autocrine mitogens. From CM we have isolated a 55-kD antiproliferative component capable of completely inhibiting SC proliferation in response to potent mitogens including regenerating axons. Results of the present study show the 55-

kD NAP to possess metalloprotease activity. The SC-derived metalloprotease exhibited biochemical, proteolytic, and immunological properties similar to those reported for stromelysin isolated from other sources (Chin et al., 1985; Umenishi et al., 1990). In addition, our SCs were found by PCR techniques to express stromelysin mRNA (personal communication; Dr. Zena Werb, University of California, San Francisco, CA). These findings suggested that the 55-kD antiproliferative factor isolated from SC CM was indeed stromelysin and directed testing showed that NAP activity was selectively depleted by anti-stromelysin antibodies.

Our results raised the possibility that autocrine control of SC proliferation involved a cascade of proteolytic activities. For most of our initial studies we used RN22 Schwannoma cultures as a source of CM and characterized NAP as a 55-kD metalloprotease. The predominant form of stromelysin immunoreactivity found in RN22 CM corresponds to the latent, 55-kD form of stromelysin indicating that activation of the metalloprotease by the schwannoma cultures is inefficient. The 55-kD proform was activated by APMA and presumably by components produced by SCs in the test cultures. The latter presumption was verified by the finding that SC CM contained predominantly the 44-kD activated form of stromelysin. In retrospect, RN22 CM was a favorable source of NAP-metalloprotease activity since the proform is more stable during fractionations to the extent that a 44-kD form of antiproliferative activity was not detected following fractionations. The proform of stromelysin was presumably activated by a second SC-derived proteolytic activity which is not found in the schwannoma cultures. It is interesting that SC cultures produce extracellular plasminogen activator (Kalderon, 1984*a*) and the plasmin-generating system has been implicated in autocrine control of proliferation and migration. Both tissue-type and urokinase-type plasminogen activators are produced by SC isolated from neonatal rat sciatic nerve (Alvarez-Buylla and Valinsky, 1985). Interestingly, the amount of the tissue-type form decreased with increasing time in culture while the production of the urokinase-type form was constitutive (Krystosek et al., 1988). Furthermore, plasmin has been reported to catalyze the first step in activation of stromelysin and this activation occurs through a urokinase-type plasminogen activator-dependent pathway (Goldberg et al., 1990). Evidently, it is possible that activation of stromelysin by SC cultures can occur through a plasminogen activator/plasmin-dependent pathway. Even though further study is required to determine any role of the plasmin-generating system in the negative control of SC proliferation involving stromelysin, it has been well established that this system is involved in the regulation of neuron–glia interactions (Kalderon, 1984*b*). In developing peripheral nerve, SCs first proliferate in response to contact with a mitogenic signal on neuronal axons and then exit the mitotic cycle and differentiate in close association with axons. We speculate that the mitogenic signal presented by neurons might modulate the proteolytic cascade by releasing a protease inhibitor or by influencing SCs to decrease the production or activation of the proenzyme.

Like stromelysin, the SC-derived, 55-kD metalloprotease cleaved FN and generated a predominant 29-kD fragment (cf., Fig. 2). Accordingly, we hypothesized that instead of acting on the cell directly, the metalloprotease cleaves FN and generates a cryptic antiproliferative activity which maintains SC quiescence. Several observations suggest that proteolysis of FN might contribute to the inhibition of SC proliferation by NAP. First, the 29-kD amino-terminal proteolytic fragment of FN possessed antiproliferative activity for SC in the nanomolar range. Second, FN is abundant in the serum added to SC culture medium and is also reportedly secreted by SCs (Cornbrooks et al., 1983). Third, SC CM (containing serum) contained a 29-kD FN fragment with antiproliferative activity.

Homandberg and co-workers (1986) have isolated and sequenced a 29-kD amino-terminal FN fragment which inhibits the in vitro proliferation of bovine aortic endothelial cells and they have made progress toward identifying the peptide sequence responsible for the antiproliferative activity (Homandberg et al., 1989). Using published procedures for obtaining well-characterized proteolytic FN fragments, we confirmed that the 29-kD amino-terminal fragment generated by other proteases also possesses antiproliferative activity for SCs. However, the antiproliferative fragment generated by the 55-kD metalloprotease was considerably more stable and probably more potent. A more detailed study of the amino acid sequences of these proteolytic preparations is required to determine the potential differences in the cleavage sites addressed by these proteases.

In the above studies we generated and assayed a variety of FN fragments and found that only the 29-kD amino-terminal fragment (CAFF) expressed potent antiproliferative activity capable of completely inhibiting proliferation by mitogen-stimulated SCs. Since intact FN increases SC proliferation (Muir et al., 1989*a*; Baron-Van Evercooren et al., 1982), it follows that the antiproliferative activity of the 29-kD amino-terminal region of the FN molecule is cryptic and requires proteolytic activation before it inhibits proliferation. Following proteolysis, the amino-terminal fragment becomes separated from the RGD cell binding domain of FN. To test directly whether the inhibitory activity of CAFF could be antagonized by native FN or RGD-containing FN fragments, mitogen-stimulated SCs were treated with a maximally inhibitory concentration of CAFF in the presence of cell-binding FN polypeptides. The presence of these FN polypeptides did not diminish the inhibitory activity of CAFF (unpublished observations) suggesting that the antiproliferative effect on SCs is not mediated by conventional FN receptors. Similarly, Homandberg and co-workers (1985) reported that inhibition of endothelial cell proliferation by an amino-terminal FN fragment was not antagonized by native FN.

In support of the hypothesis that inhibition of SC proliferation by the 55-kD metalloprotease and the 29-kD FN fragment occurs through the same pathway, we demonstrated that both proteins expressed parallel activities for normal and transformed SCs. Previously we reported (Muir et al., 1990*a*) inhibition by the 55-kD NAP to be independent of the type of mitogen used to stimulate SC proliferation, and, using the same mitogens, this observation held true for inhibition by CAFF. Also, compared to normal SCs, immortalized and transformed SC lines are nearly unresponsive to the inhibitory affects of NAP. Similarly, proliferation by immortalized SC and two schwannoma cell lines was not inhibited substantially by the antiproliferative FN fragment. It is interesting to speculate about the possible connection between the lack of response by transformed SCs and the lack of activated stromelysin found in their CM.

SC mitogens such as cholera toxin that increase intracellular cAMP require the presence of serum or growth factors (Ridley, 1990; David, 1990; Eccleston, 1989) and elevated cAMP might cause an up-regulation of growth factor receptors on SCs (Weinmaster and Lemke, 1990). Moreover, mitogenic axonal membranes have been reported to elevate cAMP (Ratner, 1984; see Meador-Woodruff et al., 1984 for contrary report) and recent speculation has raised the idea that the effects of axon-associated signals in SC development are to a significant extent mediated by elevation of cAMP levels in SCs (Jessen and Mirsky, 1991). Down-regulation of growth factor receptors might be a mechanism for antiproliferative control in the presence of diverse SC mitogens, including the axonal mitogen. Our results are compatible with the presumption that, although quiescent SCs might down-regulate growth factor receptors, they actively express receptors that mediate an autocrine antiproliferative activity (by binding the antiproliferative FN fragment). If these two opposing classes of receptors are reciprocally regulated, agents that elevate cAMP and induce receptors for growth promoters might coordinately decrease receptors for growth inhibitors. Consistent with this contention, we have established a line of SCs immortalized by prolonged treatment with cholera toxin. In addition, this SC line requires serum for continued proliferation in the absence of cholera toxin. It appears likely that, as part of a transformed phenotype, immortalized SCs and schwannoma cell lines constitutively overexpress growth factor receptors while both are unresponsive to the antiproliferative activities they secrete. It will be important to test further the implication that under expression of receptors for a cryptic FN fragment might explain how transformed SCs have escaped negative autocrine growth control.

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