**Abstract.** We found high levels of the c-src gene product in neuroendocrine tissues from adult animals. To understand the role of this proto-oncogene product, the subcellular localization of p60<sup>src</sup> was studied in neuroendocrine tissue from adrenal medulla. The results indicate that p60<sup>src</sup> was highly enriched in chromaffin granule membranes, in stable association with a protein of 38 kD. The complex with the 38-kD protein was also detected in brain, a tissue known to carry high levels of p60<sup>src</sup>. The 38-kD protein is not calpain I, II, or synaptophysin. Comparison of its peptide map showed a high degree of conservation among the different species and tissues examined. The interaction between p60<sup>src</sup> and the 38-kD protein involves disulphide bonds that are stable even when the cell fractionation is performed in the presence of a reducing agent. Since the presence of disulphide bonds among cytoplasmic proteins is very unlikely, the possibility of a noncovalent association between p60<sup>src</sup> and the 38-kD protein in vivo is discussed. The 38-kD protein may be involved in a function of p60<sup>src</sup> related to secretory organelles.

**Materials and Methods**

**Adrenal Medullae Subcellular Fractionation**

Bovine adrenal glands were obtained from a slaughter house. 20 medullae were dissected from the cortex and chromaffin granules were purified by the method of Cidon and Nelson (1983) with few modifications. The homogenization buffer (referred to as SME) contained 0.3 M sucrose, 10 mM MOPS (morpholino-ethane sulfonic acid) pH 7.5, 5 mM EDTA, 1% Trasylol, and 10 mM leupeptin and antipain. The protease inhibitors were present in all the buffers used. After homogenization, the suspension was centrifuged at 1,000 g for 15 min. The pellet was discarded and the supernatant was centrifuged at 10,000 g for 20 min. This pellet was gently resuspended in 30 ml of SME and 5 ml aliquots were directly loaded on top of sucrose layers in cellulose nitrate tubes. The bottom layer contained 15 ml of 1.8 M sucrose and the top layer contained 10 ml of 1.2 M sucrose in a buffer containing 10 mM MOPS (pH 7.5). After an overnight centrifugation in a rotor (model SW 28; Beckman Instruments Inc., Palo Alto, CA) for 20,000 rpm at 2°C, the chromaffin granules were collected from the pellet and lysed in a mini-

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**Abbreviations used in this paper:** CEF, chicken embryo fibroblasts; CGM, chromaffin granule membranes; PM, plasma membranes.
Brains of fetal rats of day 18 were processed according to Pfenninger et al. (1979) with few modifications. The 10,000-g supernatant (see above) was centrifuged in a rotor (Type 60 Ti; Beckman Instruments Inc.) at 40,000 rpm for 60 min, resuspended in SME containing 25% glycerol, quickly frozen, and stored at -70°C.

Plasma membranes (PM) were prepared by the method of Meyer and Burger (1979) with few modifications. The 10,000-g supernatant (see above) was centrifuged in a rotor (Type 60 Ti; Beckman Instruments Inc.) at 40,000 rpm for 60 min. The pellet was resuspended by a few strokes of dounce homogenization in SME buffer and mixed with 2 vol of 52% (wt/wt) sucrose (containing MOPS and EDTA as in SME buffer), to give a final concentration of 40%. 12 ml of this suspension was placed at the bottom of a cellulose nitrate centrifuge tube and overlaid with the following layers of sucrose: 14 ml of 36% (wt/wt), 8 ml of 32% (wt/wt), and 5 ml of 20% (wt/wt). The samples were centrifuged for 120 min at 131,000 rpm in a rotor (model SW 28; Beckman). The 32-36% interface was used as the source of PM and stored at -70°C. Aliquots were thawed, diluted in 10 mM MOPS (pH 7.5), and centrifuged in the Type 60 Ti rotor at 40,000 rpm for 60 min, and the PM pellets were then lysed in RIPA buffer (see below). The Percoll gradient step used by Meyer and Burger was omitted because prolonged handling of the homogenate resulted in substantial degradation of p60-α-esterase.

The total homogenate, CGM, and PM were assayed for the presence of Dopa-β-hydroxylase by Western blot with a specific antisera kindly provided by Dr. Fleming at the University of Washington (Duong and Fleming, 1984), and the data are shown in Table I. The various fractions were tested for PM-specific markers by assaying acetylcholinesterase (Elliott et al., 1986) and alkaline phosphatase specific activity (Ray, 1970). The data are summarized in Table I.

**Isolation of Nerve Growth Cones**

Brains of fetal rats of day 18 were processed according to Pfenninger et al. (1983). Briefly, the brains were homogenized in a buffer containing 0.32 M sucrose, 1 mM MgCl₂, 1 mM TES-NaOH, pH 7.3, centrifuged at 3,500 rpm for 15 min. The supernatant was spun in a Type 60 Ti rotor at 40,000 rpm for 60 rain. The pellets were resuspended in SME plus 25% glycerol and stored at -70°C.

**Chicken Brain Membranes**

Crude membranes from chicken embryo brains were prepared by differential centrifugation. 10 brains from day 14 embryos were homogenized in a Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) with low speed for 60 s in SME buffer (10% wt/vol), followed by 10 strokes of dounce homogenization. The suspension was centrifuged at 1,000 g for 15 min. The post-nuclear supernatant was centrifuged in a Type 60 Ti rotor at 40,000 rpm for 60 min, and the pellet was resuspended in a small volume and stored at -70°C.

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**Table I. Distribution of Marker Enzymes in Adrenal Medulla Subcellular Fractions**

| Sample       | Percent protein recovery | p60-α-esterase | Acetylcholinesterase | Alkaline phosphatase | Dopamine-β-hydroxylase |
|--------------|--------------------------|----------------|----------------------|----------------------|------------------------|
| Homogenate   | 100.0                    | 1.0            | 1.0                  | 1.0                  | 1.0                    |
| Plasma membranes | 1.2                   | 9.0            | 4.1                  | 19.7                 | 1.7                    |
| Chromaffin granule membranes | 4.2                 | 11.0           | 0.5                  | 1.3                  | 3.0                    |

* Relative value as compared with the amount of p60-α-esterase in the homogenate determined by densitometric scanning of the Western blot shown in Fig. 1 a.
† The relative value of specific activity is given in comparison with that of the homogenate. The specific activity in the homogenate was 4.4 μmol/s of substrate converted per h/mg of protein.
‡ As for h. The specific activity in the homogenate was 0.6 μmol of substrate converted per h/mg of protein.

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**Fractionation of Chicken Cerebella**

Cerebella were dissected from adult chicken brains, homogenized in 4 vol of SME buffer at low speed for 30 s in a Tekmar Tissumizer (Tekmar Co.), followed by 50 strokes of dounce homogenization. The suspension was centrifuged at 1,000 g for 15 min. The supernatant was spun in a Type 60 Ti rotor at 40,000 rpm for 60 min, resuspended in SME, and 1.2 ml was loaded on top of a discontinuous sucrose gradient made with 5.5 ml each of 2, 1.4, 1.2, and 0.8 M sucrose. The gradient was centrifuged in an SW 28 rotor at 21,000 rpm for 6 h (Roda et al., 1980). Fractions were collected at each interface, diluted in hypotonic buffer containing 10 mM MOPS, spun in a Type 60 Ti rotor at 40,000 rpm for 60 min, and the pellets were stored at -70°C.

**Protein Biochemistry**

Protein extracts were obtained by solubilization of tissue fractions in RIPA buffer: 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Triton-X 100, 1% sodium deoxycholate, 0.1% SDS, 1% Trasylol, and 25 mM leupeptin and antipain. The protein lysates were centrifuged at 4°C for 15 min in a microfuge. Protein concentration was measured by the Bio-Rad protein assay, using BSA as a standard. The sedimentation behavior of p60-α-esterase was analyzed in a 10-25% glycerol gradient, containing RIPA buffer without SDS. Usually 2-3 mg of protein lysate in 400 μl vol was loaded on top of a 5-ml gradient and centrifuged at 44,000 rpm for 21 h. 0.15 ml fractions were collected, and half were used for kinase assay and half for Western blot analysis.

p60-α-esterase immunoprecipitations were performed by incubation with excess mAb 327 (Lipsich et al., 1983), followed by anti-mouse Ig and protein A-Sepharose. Immunoprecipitates were washed three times with RIPA (300 mM NaCl), 1 time in RIPA (10 mM NaCl), 2 times in 40 mM Tris-HCl (pH 7.2), and once with kinase buffer, containing 20 mM Tris-HCl (pH 7.2) and 5 mM MgCl₂. The autophosphorylations reactions were performed in 20-40 μl of kinase buffer containing 10 μCi of [γ-32P]ATP (3,000 Ci/m mole; Amersham Corp., Arlington Heights, IL), to give a final ATP concentration of <0.1 μM, for 10 min at room temperature. When autophosphorylation reactions were to be analyzed by SDS-PAGE under nonreducing conditions, 0.1% Triton-X 100 was present in all the buffers to avoid p60-α-esterase aggregation. Phosphorylation of enolase was carried in 40 μl of kinase buffer containing 1 μg of enolase (Sigma Chemical Co., St. Louis, MO) denatured as described by Cooper et al. (1984), 20 μCi of [γ-32P]ATP (3,000 Ci/m mole), and 5 μM cold ATP. The reactions were stopped at various times as indicated in the figure with 20 μl of gel buffer containing 250 mM Tris-HCl (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, 0.02% bromophenol blue, and 75% glycerol. The samples were boiled for 2 min and loaded on 10% SDS-PAGE (Laemmli, 1970). For Western blot analysis gels were transferred to nitrocellulose filters, blocked in PBS containing 0.05% Tween 20 and 2.5% normal sheep serum, incubated overnight with mAb 327 at room temperature, and washed in PBS/Tween 20 with several buffer changes. Filters were preincubated in the same solution containing 2.5% sheep serum for 30 min to 1 h, followed by the addition of 10 μCi of [32P]I.
A 38-kD protein was phosphorylated in the immunocomplex with three different mAb directed against separate epitopes of p60<sup>-src</sup> (Lipsich et al., 1983; Parsons et al. 1984; Fig. 2 a, lanes 1-4). Controls immunoprecipitations with anti-mouse immunoglobulins did not show any phosphorylated products (Fig. 2 a, lane 5).

5-7). A 38-kD protein was phosphorylated in the immunocomplex with three different mAb directed against separate epitopes of p60<sup>-src</sup> (Lipsich et al., 1983; Parsons et al. 1984; Fig. 2 a, lanes 1-4). Controls immunoprecipitations with anti-mouse immunoglobulins did not show any phosphorylated products (Fig. 2 a, lane 5).
In vitro kinase assays using antisera directed against known proteins of similar molecular weight also present in adrenal medulla, such as synaptophysin (Jahn et al., 1985; Navone et al., 1986; Wiedenmann and Franke, 1985), the p36 substrate of p60<sup>56</sup>-specific antibodies (Lipsich et al., 1983; lanes 2 and 3; GD11 and EB8 also directed vs. p60<sup>56</sup> (Parsons et al., 1984, 1986); lane 5, anti-mouse Ig; lane 6, polyacrylamide serum anti-

Figure 2. (a) Kinase assays were performed as above on 200 μg of CGM protein lysate using various antibodies. Lanes 1 and 4, mAb 327 and 273 specific for p60<sup>56</sup>-specific antibodies (Lipsich et al., 1983); lanes 2 and 3, GD11 and EB8 also directed vs. p60<sup>56</sup> (Parsons et al., 1984, 1986); lane 5, anti-mouse Ig; lane 6, polyacrylamide serum anti-

synaptophysin; lane 7, polyacrylamide serum against bovine-derived cal-
pactin I; lane 8, polyacrylamide serum against bovine-derived calpactin II. (b) Western blot analysis. Lanes 1, 4, and 7, 100 μg of postnuclear supernatant of adrenal medulla; lanes 2, 5, and 8, 100 μg of CGM; lanes 3, 6, and 9, 400 μg of CGM immunoprecipitated with mAb 327. mAb 327 was used to probe lanes 1-3, anti-calpactin I for lanes 4-6, and anti-calpactin II for lanes 7-9.

The presence of the 38-kD protein (p38) in the immunocomplex with anti-p60<sup>56</sup>-specific antibodies suggested that p60<sup>56</sup> might be stably complexed to this protein in CGM. We therefore analyzed the sedimentation profile of p60<sup>56</sup> from protein extracts of PM and CGM fractions using a 10–25% glycerol gradient. Under these conditions the molecular mass markers ranging from 26 to 200 kD were separated (Fig. 3 e). Each fraction from the gradient was divided into two parts: one was directly analyzed by Western blot with mAb 327 (Fig. 3, c and d), and the other was used in a kinase assay performed after immunoprecipitation with mAb 327 (Fig. 3, c and d). Densitometric scanning of the Western blots showed that p60<sup>56</sup> from CGM is distributed in a broad peak shifted to a more dense region of the gradient than p60<sup>56</sup> from PM or from chicken embryo fibroblasts (CEF) infected with a recombinant Rous sarcoma virus, NY55, which overexpresses p60<sup>56</sup> (Levy et al., 1986; Fig. 4, A and B). The kinase assay across the gradient of CGM showed a 38-kD protein in the fractions that coincide with the fast sedimenting p60<sup>56</sup> molecules detected by Western blot analysis (Fig. 3 d), suggesting a physical association between the two proteins. The distribution of monomer and complexed forms of p60<sup>56</sup> usually overlaps in the glycerol gradient rendering the visualization of the two peaks detected by Western blot as a single broader peak. We predicted, from the distribution of p38 phosphorylation that the molecular mass of the complex would be between 120 and 200 kD. Further analysis of the complex will more precisely define this point (see below).

The p38 band appeared as a doublet when lower percentage acrylamide gels were used (Fig. 3 d). A small fraction of p60<sup>56</sup> in PM also seemed to be complexed with p38 as detected in a kinase assay across the gradient (Fig. 3 c). The phosphorylated 52–54-kD doublet that localizes in the same region of the gradient as the p38/p60 complex (Fig. 3 d) was shown by peptide mapping to be a degradation product of p60<sup>56</sup> (data not shown). Overexposure of Western blots of the same gradients did not show an enrichment of the 52–54-
Figure 3. Glycerol gradient sedimentation profile of p60-src from PM and CGM lysates. 10–25% gradient was separated into 30 fractions of 150 µl each and 75 µl was used for Western blot analysis with mAb 327. PM and CGM in a and b, respectively. The other half was immunoprecipitated with mAb 327 followed by kinase assay as described in the legend to Fig. 1. PM and CGM in c and d, respectively. The numbers on top of each lane correspond to the fraction numbers from the lightest to the heaviest regions of the gradient (direction of sedimentation is left to right). (e) Sedimentation profile of 14C-labeled molecular mass standards in a parallel gradient. (f) Western blot analysis with mAb 327 of CGM lysates separated on glycerol gradient in the absence (top) or in the presence of 5 mM DTT (bottom).
kD degradation product in the region of the complex; it is possible that there is a preferential phosphorylation of these p60src proteolytic products present in the complex with p38 relative to the intact p60src bound to p38.

**Stability of the Complex and Disulphide Bonds**

The p38/p60 complex in RIPA buffer was stable in the presence of 2% SDS, 1 M NaCl, and 5 mM EGTA (data not shown), but was dissociated by the presence of 5 mM dithiothreitol (DTT). Under this condition the p60src sedimentation profile, analyzed by Western blot, collapsed to a narrower peak shifted to the left (Figs. 3f and 4C). Thus the stability of the complex is likely to be dependent on disulphide bridges.

Phosphorylation of anti-p60src immunoprecipitates and analysis of the p38/p60 complex by nonreducing SDS-PAGE followed by autoradiography showed a broad band ranging from 110 to 140 kD (Fig. 5a, lane 2). p38 was absent, and a decrease in intensity of p60 was detectable. Lane 1 of Fig. 5 shows, as molecular mass reference, the typical pattern of p38/p60 complex analyzed under reducing conditions, but is not directly comparable with lane 2 since they represent two different CGM preparations. The broad 110-140-kD band was split into upper and lower bands, excised from the gel, incubated in sample buffer containing 30 mM DTT, and analyzed by SDS-PAGE. p38 and p60 are then visualized as separated proteins (Fig. 5b, lanes 1 and 2). No major differences were found between the upper (lane 1) and lower (lane 2) portion of the band, except for a more intense signal of p38 in lane 1. This experiment confirms the p60src glycerol gradient sedimentation data and indicates that the complex could contain either one or two p38 molecules directly linked via disulphide bonds to p60src. The relatively low level of p60 phosphorylation detected in Fig. 5b suggests that in vitro autophosphorylation in the complex might be suppressed. The 65-kD band, which was more prominent under nonreducing conditions, (Fig. 5a, lanes 2 and 3) was also analyzed in the presence of DTT and found to comigrate with p60src (Fig. 5b, lane 6). A 65-kD band is also detected when iodoacetamide is included in the homogenization buffer (see below).

Disulphide bonds are rarely found among cytoplasmic proteins due to the high concentration of glutathione in the cell (Barron and Singer, 1943). We therefore examined whether or not the p38/p60 complex could be detected in chromaffin granules isolated in the presence of a reducing or alkylating agent. The presence of 1 mM 2-mercaptoethanol throughout the cell fractionation did not interfere with the detection of the complex (Figs. 5a, lane 3, and 6a, lane 2). Similarly, overnight exposure of isolated chromaffin granules to 1 mM 2-mercaptoethanol did not affect the detection of the complex (Fig. 6b, lane 2). Conversely, 1 h exposure of CGM RIPA lysates to 1 mM 2-mercaptoethanol promptly caused disruption of p38/p60 complex (Fig. 6c, lane 2). These results imply that under native conditions either membrane components and/or the protein conformation itself confer on the complex resistance to reducing agents. Even in the presence of 2-mercaptoethanol, disulphide bonds are present within the complex (Fig. 5a, lane 3).

Similar experiments were performed using 10 mM iodoacetamide. When iodoacetamide was included in the homog-

Figure 4. Quantitation by densitometry of the distribution of p60src in glycerol gradients analyzed by Western blotting with mAb 327. (A) The data displayed by • show the profile of p60src from CEF overexpressing the c-src gene from a recombinant retroviral vector (Levy et al., 1986). Although a small fraction of p60src in CEF is complexed with p38 as detected in kinase assay across the gradient (data not shown), the majority is in a free form. The data represented by • show the sedimentation profile of p60src from PM of adrenal medulla. (B) ○, control as in A. •, sedimentation profile of p60src from CGM (the data of the PM and CGM are averages of two independent cell fractionation experiments). (C) Dissociation of the complex by 5 mM DTT. •, profile of p60src from CGM. △, the same lysate separated on the gradient in the presence of DTT. The direction of sedimentation is left to right.
Figure 5. Nonreducing SDS-PAGE analysis of p38/p60 complex. (a) Immunoprecipitates with mAb 327 of CGM RIPA lysates were phosphorylated in a kinase assay, boiled in sample buffer with or without reducing agents (lanes 1 and 2, respectively), and analyzed by 7.5% SDS-PAGE. Lane 3 shows also nonreducing SDS-PAGE analysis of immunoprecipitates of CGM lysates from chromaffin granules purified in the presence of 1 mM 2-mercaptoethanol. (b) The 110-140-kD band of a, lane 2, split into upper and lower bands was analyzed by 10% SDS-PAGE after incubation with 30 mM DTT (lanes 1 and 2, respectively). Lanes 3 and 4, as reference, show the migration of p38 and p60, respectively, upon excision of the corresponding bands from a reducing gel. Lanes 5 and 6 show the migration of p60 and the 65-kD band from a under reducing conditions.

Figure 6. Effects of 1 mM 2-mercaptoethanol on the stability of the complex. (a) Adrenal medulla cell fractionation was performed in parallel without (lane 1) or with (lane 2) 2-mercaptoethanol until the isolation of chromaffin granules. CGM protein extracts were obtained by lysis in RIPA buffer without 2-mercaptoethanol. Immunoprecipitates with mAb 327 and kinase assays were performed as previously described. (b) Chromaffin granules were isolated without 2-mercaptoethanol and then incubated overnight at 4°C without (lane 1) or with (lane 2) 1 mM 2-mercaptoethanol, and analyzed as above. (c) CGM RIPA lysates were immunoprecipitated without (lane 1) or with (lane 2) 1 mM 2-mercaptoethanol, washed as usual in the absence of 2-mercaptoethanol and phosphorylated by kinase assay.

In Vitro Kinase Activity of p60\textsuperscript{src} in the Complex

To determine whether the specific activity of the p60\textsuperscript{src} protein kinase is changed by complex formation, fractions from the glycerol gradient corresponding to either the monomer or complex form of p60\textsuperscript{src} were pooled, and the specific activity of the kinase was determined by an in vitro kinase assay with enolase as an exogenous substrate and by measurement of the amount of p60\textsuperscript{src} by Western blot. As shown in Fig. 8, no difference was found between the two fractions in their ability to phosphorylate enolase when the two fractions were normalized for the amount of p60\textsuperscript{src} (data not shown). We conclude that there is no generalized increase in kinase activity of the complexed p60\textsuperscript{src} relative to the monomer in vitro. In addition, the results indicate that p38 binding to p60\textsuperscript{src} does not impair the ability of the enzyme to phosphorylate an exogenous substrate.
Figure 7. Effects of iodoacetamide on the stability of the complex. (a) Adrenal medulla cell fractionation was performed in parallel without (lanes 1 and 3) or with (lanes 2 and 4) 10 mM iodoacetamide. RIPA lysis of the CGM were also in the presence of iodoacetamide, immunoprecipitates were washed as usual, subjected to kinase assay, and analyzed by SDS-PAGE under reducing (lanes 1 and 2) and nonreducing (lanes 3 and 4) conditions. (b) Chromaffin granules were isolated without iodoacetamide and then exposed overnight at 4°C without (lane 1) or with (lane 2) 1 mM iodoacetamide. RIPA lysates of CGM, immunoprecipitations, and kinase assays were performed as usual. Lanes 3 and 4 show the effect of 1 mM iodoacetamide added during the RIPA lysis (lane 3 is the control; lane 4 with iodoacetamide) to chromaffin granules isolated without iodoacetamide. Immunoprecipitates were washed without iodoacetamide and processed as above.

As a control of our assay procedure, we performed the same experiment with cell lysates from CEF transformed by a retroviral vector expressing middle T antigen (Kornbluth et al., 1986), a viral protein known to increase the kinase activity of p60οκ'οκ (Bolen et al., 1984; Courtneidge, 1985). In agreement with the previous studies, we observed an increase in the kinase activity of the fraction of p60οκ'οκ bound to middle T antigen relative to the free form (data not shown).

Although p38 does not inhibit in vitro phosphorylation of enolase, it does partially inhibit autophosphorylation of p60οκ'οκ. This effect can be observed by comparison of Fig. 3, b and d where the amount of p60οκ'οκ detected by Western blot is equivalent in the monomer and complexed form, yet the phosphorylation signal of the complexed p60οκ'οκ is less relative to the monomer. Conversely, the phosphorylation of the 52–54-kD degradation product is not inhibited. The inhibition of autophosphorylation is reversible as can be seen upon disruption of the complex by 1 mM 2-mercaptoethanol.

Figure 8. Comparison of the kinase activity of free vs. complexed p60οκ'οκ. (a) Fractions numbered 10, 11, 12, 13 (free) and 17, 18, 19, 20 (complexed), obtained by glycerol gradient sedimentation, were pooled and immunoprecipitated with mAb 327. They were then divided into six tubes: one was used for Western blot analysis (b) and the others were incubated in 40 μl of kinase buffer containing 1 μg of enolase, 5 μM cold ATP, and 20 μCi of [γ-32P]ATP (3,000 Ci/m mole) for 2, 5, 10, and 20 min, as indicated on the bottom of each lane, at room temperature. The reactions were stopped with electrophoresis buffer, and the samples were boiled for 2 min and then separated on a SDS-PAGE. To decrease the background of unincorporated labeled ATP, the gel was treated with alkali (Cooper et al., 1983) and autoradiographed. (b) Part of the immunoprecipitate described above was directly loaded onto a gel in electrophoresis buffer without boiling so that the mAb 327 Ig chains remained near the top of the gel. After electrophoresis the gel was transferred to nitrocellulose, incubated with mAb 327 followed by 125I-labeled sheep anti-mouse Ig, and autoradiographed.

p38/p60 Complex Is Also Present in Brain

p60οκ'οκ expression is particularly high in the developing embryonic brain where immunocytochemical studies have localized it to neuronal processes (Fults et al., 1985; Maness, 1986), suggesting a participation of p60οκ'οκ in neuronal differentiation and axonal growth. Rat brains from 18-d-old embryos were fractionated on a sucrose gradient according to Pfenninger et al. (1983) and each fraction was tested by kinase assay and Western blot analysis. We found a twofold enrichment of p60οκ'οκ relative to the total homogenate in the subcellular fraction enriched for growth cones, a highly specialized portion of the postmitotic differentiating neuron that contains clusters of vesicles likely to participate in the growth of neurites (Fig. 9 a, lanes 1 and 3, respectively). The corresponding kinase assay revealed a prominent p38 both in the growth cone fraction and, to a lesser extent, in the heavier membrane fraction (Fig. 9 b, lanes 3 and 4), suggesting that a complex similar to that of adrenal medulla is also present in embryonic brain. V8 protease mapping indicated that p38 from bovine adrenals and rat brain are almost identi-


**Discussion**

In the present study we have shown that p60<sup>c-src</sup> is enriched in CGM isolated from bovine adrenal medullae and is complexed with a 38-kD protein. The enrichment of p60<sup>c-src</sup> kinase activity in chromaffin granules was previously described by Parsons and Creutz (1986). We have confirmed and extended their data by showing that the increase in kinase activity is due to a parallel enrichment of p60<sup>c-src</sup> protein as detected by Western blot analysis, rather than an increase in the specific activity of the protein. Although the relative enrichment of p60<sup>c-src</sup> is similar in both CGM and PM compartments, the total surface area of the granule membranes is ~10 times greater than that of the plasma membrane (Phillips and Pryde, 1987), suggesting that a large pool of the total p60<sup>c-src</sup> is found in the granule membrane compartment. The 38-kD protein complexed with p60<sup>c-src</sup> in CGM is different from calpain I and II and synaptophysin. Several proteins of similar molecular masses, abundant in the tissues examined, have been identified and represent possible candidates for the identity of p38. Chromobindins for example, which include calpain I and II, are a family of related proteins characterized by their calcium-dependent binding to CGM (Creutz et al., 1987). GTP binding proteins of 39–40 kD have also been purified from chromaffin granules (Toutant et al., 1987). Two-dimensional gel analysis of the phosphorylated p38 showed that it is an acidic protein (data not shown), thereby suggesting no relations with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a basic protein that can be in vitro phosphorylated on tyrosine by epidermal growth factor receptor (Reiss et al., 1986). At present we do
not have data supporting similarities between p38 and other known proteins.

The p38/p60 complex was detected also when adrenal medullae were lysed directly with RIPA buffer. We found that the complex contains disulphide bonds even when the cell fractionation is performed in the presence of 1 mM 2-mercaptoethanol. However, the same concentration of reducing agent is sufficient to disrupt the complex if added in the presence of detergents (RIPA buffer). A close interaction between p38 and p60-src in the CGM could explain the inaccessibility of the complex to reducing agents when either protein conformation or membrane integrity is preserved (absence of detergents). Our assays of p38 binding to p60-src involve immunoprecipitation followed by a kinase assay or the sedimentation of p60-src through glycerol gradients. Both assays involve prolonged exposure to detergents. Under these conditions we detect a complex whose stability is dependent on disulphide bonding. Reconstitution experiments with the purified components, p38 and p60-src, would allow testing under controlled conditions of the parameters that may influence the complex formation and stability. The use of iodoacetamide in the homogenization buffers completely abolishes the detection of the complex, although it does not block the autophosphorylation of p60-src. It is possible that iodoacetamide disrupts the complex by altering protein structure and preventing disulphide bond formation. We hypothesize that p38 and p60-src interact noncovalently in vivo, and spontaneous disulphide bonding occurs promptly upon cell lysis.

The detection of interchain disulphide bonds among cytoplasmic proteins has been previously reported upon purification of the two protomers of the regulatory subunit of cAMP-dependent protein kinase I (Zick and Taylor, 1982). Similar to the p38/p60 complex, the disulphide bond is present even when the purification is performed in the presence of a reducing agent. Another related phenomenon has been described for the cAMP-dependent kinase II and its regulatory subunit. The two purified molecules can spontaneously form specific disulphide bonds upon in vitro incubation (First et al., 1988). Analogously, binding of p38 to p60-src may have a regulatory role.

Is p38 a substrate of p60-src in vivo? After labeling of primary adrenal cell cultures in vivo with 32P-orthophosphate followed by immunoprecipitation with mAb 327 or antiphosphotyrosine antisera, we could not detect a prominent 38-kD phosphoprotein (data not shown). Because of the possible limitations of the detection with anti-phosphotyrosine antibody, the availability of a specific antibody to p38 will allow a more precise investigation.

The subcellular localization data also suggest that p38 may be involved in anchoring p60-src to chromaffin granules, and it could perform an analogous function in other tissues as well. Alternatively, p38 might be a modulatory protein of p60-src activity. One protein that has been shown to stably associate with p60-src is middle T antigen of polyoma virus which forms a complex with p60-src in polyoma-transformed cells (Courtneidge and Smith, 1983, 1984). The association of middle T antigen increases the kinase activity of p60-src (Bolen et al., 1984; Courtneidge, 1985). In contrast, the p60-src complexed with p38 showed no increase in kinase activity under the conditions used in our experiments. It is possible that the substrate specificity of p60-src is altered by complex formation or that the activation occurs transiently, under appropriate stimuli. Binding of p38 to p60-src causes a decrease in the ability of p60-src, under the conditions used in the kinase assay, to phosphorylate itself. The influence of p38 on autophosphorylation may be a consequence of its binding to the COOH-terminal region of p60-src, thereby impinging on the autophosphorylation site.

The subcellular localization of the complex in chromaffin granules, seems to suggest that the function of p60-src in adrenal medulla is linked to the exocytotic machinery, and it is possibly influenced by p38. A very similar p38 protein, perhaps identical, is also found complexed to p60-src in embryonic rat and chicken brains and is enriched in growth cones. Although a stimulus-coupled exocytotic response has not been well-characterized in growing neurites, the growth of axonal membranes seems to occur by fusion of membrane vesicles transported from the Golgi apparatus to the growth cone (Pfenninger, 1987). Thus the p38/p60 complex could play a role in this exocytotic-like process as well.

Preliminary experiments indicated that p60-src is also abundant in pituitary gland, another neuroendocrine tissue, and a large fraction is complexed with p38. In contrast, in human platelets p38 was not detectable and a fraction of p60-src is complexed with a 150-kD protein (unpublished results). This complex also was sensitive to reducing agents. With the exception of platelets, the association of p60-src with p38 seems to occur in a number of tissues where p60-src is abundant and may play an essential role in p60-src function in vivo.

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