$p60^{c\text{-}src}$ Is Complexed with a Cellular Protein in Subcellular Compartments Involved in Exocytosis

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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 $p60^{c\text{-}src}$ was studied in neuroendocrine tissue from adrenal medulla. The results indicate that $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$. The 38-kD protein is not calpactin 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ and the 38-kD protein in vivo is discussed. The 38-kD protein may be involved in a function of $p60^{c\text{-}src}$ related to secretory organelles.

THE proto-oncogene product $p60^{c\text{-}src}$, like its viral homologue $p60^{v\text{-}src}$, has a tyrosine-specific protein kinase activity. Unlike transmembrane receptor-type protein kinases, $p60^{c\text{-}src}$ and its close relatives are membrane-associated proteins lacking transmembrane domains (reviewed in Hunter and Cooper, 1985). While the activity of the receptor-like kinases is modulated by the interaction of the extracellular moiety with appropriate ligands (Carpenter et al., 1979; Kasuga et al., 1982; Nishimura et al., 1982), no specific cellular proteins have been identified that bind to $p60^{c\text{-}src}$ and modulate its kinase activity. Possible mechanisms of regulation of this enzyme include phosphorylation (Courtneidge, 1985; Cooper and King, 1986) and , in some tissues, alternative splicing (Levy et al., 1987; Martinez et al., 1987).

$p60^{c\text{-}src}$ expression is detectable in many tissues but is considerably higher in developing nervous tissues where its appearance coincides with the end of the proliferative stage and the beginning of differentiation and morphogenesis (Cotton and Brugge, 1983; Sorge et al., 1984*a*,*b*; Fults et al., 1985). The protein is still detectable, albeit at lower levels than embryonic brain, in adult brains (Sorge et al., 1984*a*; Sudol et al., 1988). Platelets are also known to contain relatively high levels of $p60^{c\text{-}src}$ (Golden et al., 1986).

In the course of preliminary studies on the tissue distribution of $p60^{c\text{-}src}$, we found that neuroendocrine cells of adult animals also contain high levels of $p60^{c\text{-}src}$. In particular, bovine adrenal medullae express levels of $p60^{c\text{-}src}$ comparable to that of $p60^{v\text{-}src}$ in chicken embryo fibroblasts infected by Rous sarcoma virus. Since neuroendocrine tissues are specialized for secretion, we examined the possibility that $p60^{c\text{-}src}$ was enriched in secretory organelles. We have found that a large fraction of $p60^{c\text{-}src}$ in adrenal medulla is localized in chromaffin granule membranes. An enrichment of $p60^{c\text{-}src}$-related kinase activity in bovine chromaffin granules has also been described by Parsons and Creutz (1986). In this study, we show that $p60^{c\text{-}src}$ is specifically located in chromaffin granule membranes (CGM)[1] as a complex with a 38-kD protein and that the reported enrichment of kinase activity is due to a parallel enrichment of $p60^{c\text{-}src}$ protein. The complex with the 38-kD protein was also found in brain, both of embryonic and adult origin.

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 μM 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-

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblasts; CGM, chromaffin granule membranes; PM, plasma membranes.

mal volume of hypotonic buffer containing 10 mM MOPS (pH 7.5) by dounce homogenization with a tight pestle and then diluted in 500 ml of the same buffer and centrifuged at 3,000 *g*. CGM were recovered from the supernatant by centrifugation 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 a 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 $p60^{c\text{-}src}$.

The total homogenate, CGM, and PM were assayed for the presence of Dopa-β-hydroxylase by Western blot with a specific antiserum 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 (Ellman et al., 1961) 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 $MgCl_2$, 1 mM TES-NaOH, pH 7.3, centrifuged at 3,500 rpm in a rotor (model SS34; Sorvall Instruments Div., Newton, CT) for 15 min, and 11 ml of the supernatant was loaded on top of a discontinuous sucrose gradient containing 7 ml of 2.66 M sucrose, 8 ml of 1 M sucrose, 10 ml of 0.75 M sucrose. The gradients were spun at 45,000 rpm for 50 min in a vertical rotor (model VTi50; Beckman Instruments, Inc., Palo Alto, CA). The fractions at the 0.32–0.75 M and 0.75–1.0 M interface were collected, diluted in the homogenization buffer, and centrifuged in a rotor (Type 60 Ti; Beckman) at 40,000 rpm for 60 min. The pellets were resuspended in a small volume 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) at 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 postnuclear supernatant was centrifuged in a Type 60 Ti rotor at 40,000 rpm for 60 min. The pellet was resuspended in a minimal volume of 10 mM MOPS (pH 7.5) by 10 strokes of dounce homogenization, diluted to 40 ml of the same buffer, and centrifuged again as above. The pellet was resuspended in SME plus 25% glycerol and stored at −70°C.

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, 1.6, 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 μM leupeptin and antipain. The protein lysates were clarified by centrifugation 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 $p60^{c\text{-}src}$ 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.

$p60^{c\text{-}src}$ 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 $MgCl_2$. The autophosphorylations reactions were performed in 20–40 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmole; 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 $p60^{c\text{-}src}$ 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-^{32}P]ATP (3,000 Ci/mmole), 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 ^{125}I-

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

	Percent protein recoveries	$p60^{c\text{-}src}$*	Acetylcholin-esterase‡	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 $p60^{c\text{-}src}$ 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 μmols of substrate converted per h/mg of protein.
§ As for b. The specific activity in the homogenate was 0.6 μmols of substrate converted per h/mg of protein.
‖ Relative value as compared with the amount of enzyme in the homogenate determined by densitometric scanning of Western blots with anti-Dopamine-β-hydroxylase-specific antiserum kindly provided by Dr. P. Fleming (Duong and Fleming, 1984).

labeled sheep anti-mouse Ig (750–3,000 Ci/mmole; Amersham Corp.). After 2 h, the filters were washed with several changes of PBS/Tween and exposed. Quantitation was carried out by exposing gels and filters for various lengths of time without intensifying screens and scanning with a densitometer.

Peptide and Phosphoamino Acid Analysis

Limited proteolysis with *Staphylococcus aureus* V8 protease and chymotrypsin were performed as previously described (Cleveland et al., 1977; Collett et al., 1979), using 200 ng and 50 μg of enzyme, respectively, per lane in a 15% SDS-PAGE. One-dimensional phosphoamino acid analysis was performed as described previously (Hunter and Sefton, 1980) by electrophoresis of samples on cellulose thin-layer plates at pH 3.5.

Results

p60$^{c\text{-}src}$ Distribution in Adrenal Medulla

To determine the subcellular localization of p60$^{c\text{-}src}$ in adrenal medulla, cell fractionation experiments were performed on bovine glands. PM were isolated by a modification of the procedure described by Meyer and Burger (1979), and chromaffin granules were purified by the method of Cidon and Nelson (1983). Proteins were solubilized in RIPA buffer from each subcellular fraction and the levels of p60$^{c\text{-}src}$ were determined by Western blot analysis with mAb 327 (Lipsich et al., 1983). p60$^{c\text{-}src}$ was enriched 9- and 11-fold, respectively, in PM and CGM relative to the total homogenate (Fig. 1 *a*, lane *2–4*, and Table I). Table I shows the distribution of specific markers for the PM and CGM, together with the distribution of p60$^{c\text{-}src}$ as determined by densitometric scanning of the Western blot shown in Fig. 1 *a*. About 1 and 4% of total protein were recovered in PM and CGM, respectively, relative to the total cell homogenate (Table I). This means that a large pool (>40%) of p60$^{c\text{-}src}$ molecules are in the CGM compartment and a smaller fraction (~10%) is present in the plasma membrane. These are minimal estimates because of loss of material during the fractionation procedures.

p60$^{c\text{-}src}$ Is Complexed with a 38-kD Protein

When PM and CGM from bovine adrenal medullae were analyzed by a kinase assay in an immunecomplex with mAb 327, 10- and 14-fold enrichments were detected in PM and CGM fractions, respectively. Therefore, the enrichment of protein kinase activity assayed by autophosphorylation parallels the enrichment of protein detected by Western blot analysis. In addition, a 38-kD protein coprecipitated with p60$^{c\text{-}src}$ and was heavily phosphorylated in the in vitro kinase assay, particularly in the CGM samples (Fig. 1 *b*, lanes *4*, *6*, and *8*). Longer exposure of kinase assays performed on the total cell homogenate or RIPA buffer lysates of adrenal medullae also allowed the detection of a 38-kD protein (Fig. 1 *b*, lanes *5* and *7*), indicating that the coprecipitation of the 38-kD with p60$^{c\text{-}src}$ was not a consequence of the cell fractionation procedure. Phosphoamino acid analysis showed phosphotyrosine to be the only labeled amino acid in this protein (data not shown). Western blot analysis of a CGM preparation with mAb 327 did not detect a 38-kD protein (Fig. 1 *a*); indicating that the antibody could not recognize this protein directly. V8 protease peptide mapping results conclusively showed that the 38-kD protein is not a degradation product of p60$^{c\text{-}src}$ (see Fig. 10 *a*, compare lanes *1–3* and *5–7*). A 38-kD protein was phosphorylated in the immunecomplex with three different mAb directed against separate epitopes of p60$^{c\text{-}src}$ (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*).

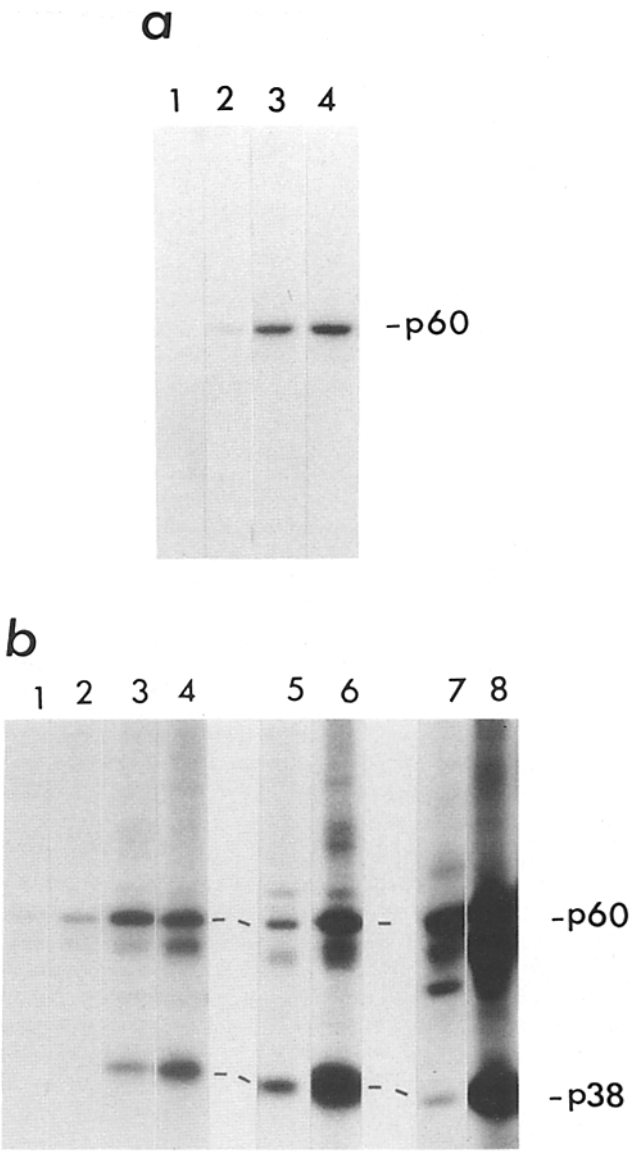

Figure 1. p60$^{c\text{-}src}$ and p38 distribution in bovine adrenals. (*a*) For Western blot analysis, 50 μg of protein lysate was resolved by SDS-PAGE, transferred to nitrocellulose, incubated with mAb 327 and then ^{125}I-labeled sheep anti-mouse Ig, and autoradiographed. Sources of proteins were (lane *1*) postnuclear supernatant of adrenal cortex and (lane 2) adrenal medullae, (lane *3*) PM from adrenal medullae and (lane *4*) CGM. (*b*) For kinase assays, 400 μg of protein lysate was immunoprecipitated with mAb 327, incubated in 30 μl of kinase buffer with 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), washed twice in RIPA buffer, and analyzed by SDS-PAGE and autoradiography. Lanes *1–4* are labeled as in *a* above. Lanes *5* and *6* show a longer exposure of a kinase assay with mAb 327 of postnuclear adrenal medullae homogenate and CGM, respectively. Lane *7*, kinase assay of a direct RIPA lysate of adrenal medullae; lane *8*, kinase assay of CGM RIPA lysate as control.

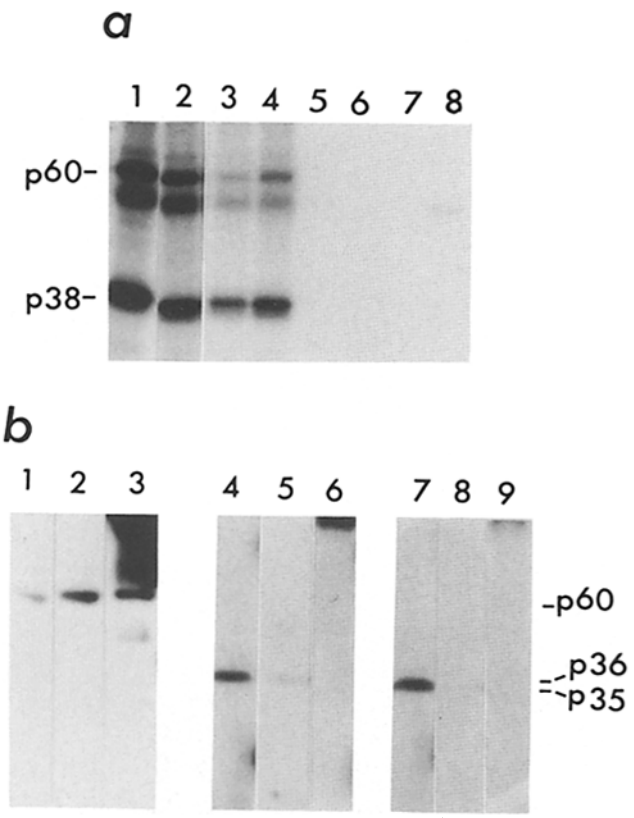

Figure 2. (*a*) Kinase assays were performed as above on 200 μg of CGM protein lysate using various antibodies. Lanes *1* and *4*, mAb 327 and 273 specific for $p60^{c\text{-}src}$ (Lipsich et al., 1983); lanes *2* and *3*, GD11 and EB8 also directed vs. $p60^{c\text{-}src}$ (Parsons et al., 1984, 1986); lane *5*, anti-mouse Ig; lane *6*, polyclonal serum anti-synaptophysin; lane *7*, polyclonal serum against bovine-derived calpactin I; lane *8*, polyclonal 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*.

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 $p60^{v\text{-}src}$ (also named calpactin I; Radke et al., 1980; Erikson and Erikson, 1980; Radke et al., 1983; Glenney, 1986; Huang et al., 1986; Creutz et al., 1987), and p35, the substrate of epidermal growth factor receptor (also named calpactin II; Glenney, 1986), did not reveal a phosphorylated 38-kD protein (Fig. 2 *a*, lanes *6–8*). Since the antisera used for immunoprecipitations could in principle block the phosphorylation reaction, the following experiment was performed. CGM lysed in RIPA buffer were immunoprecipitated with mAb 327, separated by SDS-PAGE, and transferred to nitrocellulose. The blots were incubated in parallel with either mAb 327 or anti-calpactin I or II sera. No immunoreactivity was detected in the immunoprecipitate with either calpactin I or II (Fig. 2 *b*, lanes *6* and *9*), although strong signals were detected in the whole adrenal medulla homogenate by these antibodies (Fig. 2 *b*, lanes *4* and *7*). To directly compare the peptide maps of these proteins with p38, phosphorylated calpactin I was obtained by in vivo labeling with ^{32}P-orthophosphate of chicken embryo fibroblasts transformed by Rous sarcoma virus, followed by immunoprecipitation with specific antisera (Radke et al., 1983). The phosphorylation pattern in vivo and in vitro of calpactin I are the same (Erikson and Erikson, 1980). Synaptophysin was obtained by in vitro phosphorylation of purified rat brain synaptic vesicles (kindly provided by Dr. Dennis Pang and Dr. Paul Greengard, Rockefeller University, New York) and under those conditions synaptophysin is exclusively phosphorylated on tyrosine (Pang et al., 1988). The phosphopeptide patterns obtained by partial cleavage with V8 protease and chymotrypsin of calpactin I (see Fig. 10 *a*, lane *4*) and synaptophysin (see Fig. 10 *b*, lane *3* and Fig. 10 *c*, lane *3*) were clearly different from that of the phosphorylated 38-kD protein present in CGM (see Fig. 10 *a*, *b*, and *c*, lanes *1*). These differences cannot be attributed to species differences since a comparison of the 38-kD protein within the same species, with calpactin I (see Fig. 10 *a*, compare lanes *3* and *4*) or synaptophysin (see Fig. 10, *b* and *c*, compare lanes *2* and *3*) showed that they do not share any common in vitro phosphorylated peptide.

The presence of the 38-kD protein (p38) in the immunecomplex with anti-$p60^{c\text{-}src}$-specific antibodies suggested that $p60^{c\text{-}src}$ might be stably complexed to this protein in CGM. We therefore analyzed the sedimentation profile of $p60^{c\text{-}src}$ from protein extracts of PM and CGM fractions using a 10–25% glycerol gradient. Under these conditions 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, *a* and *b*), 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 $p60^{c\text{-}src}$ from CGM is distributed in a broad peak shifted to a more dense region of the gradient than $p60^{c\text{-}src}$ from PM or from chicken embryo fibroblasts (CEF) infected with a recombinant Rous sarcoma virus, NY5H, which overexpresses $p60^{c\text{-}src}$ (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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ (data not shown). Overexposure of Western blots of the same gradients did not show an enrichment of the 52–54-

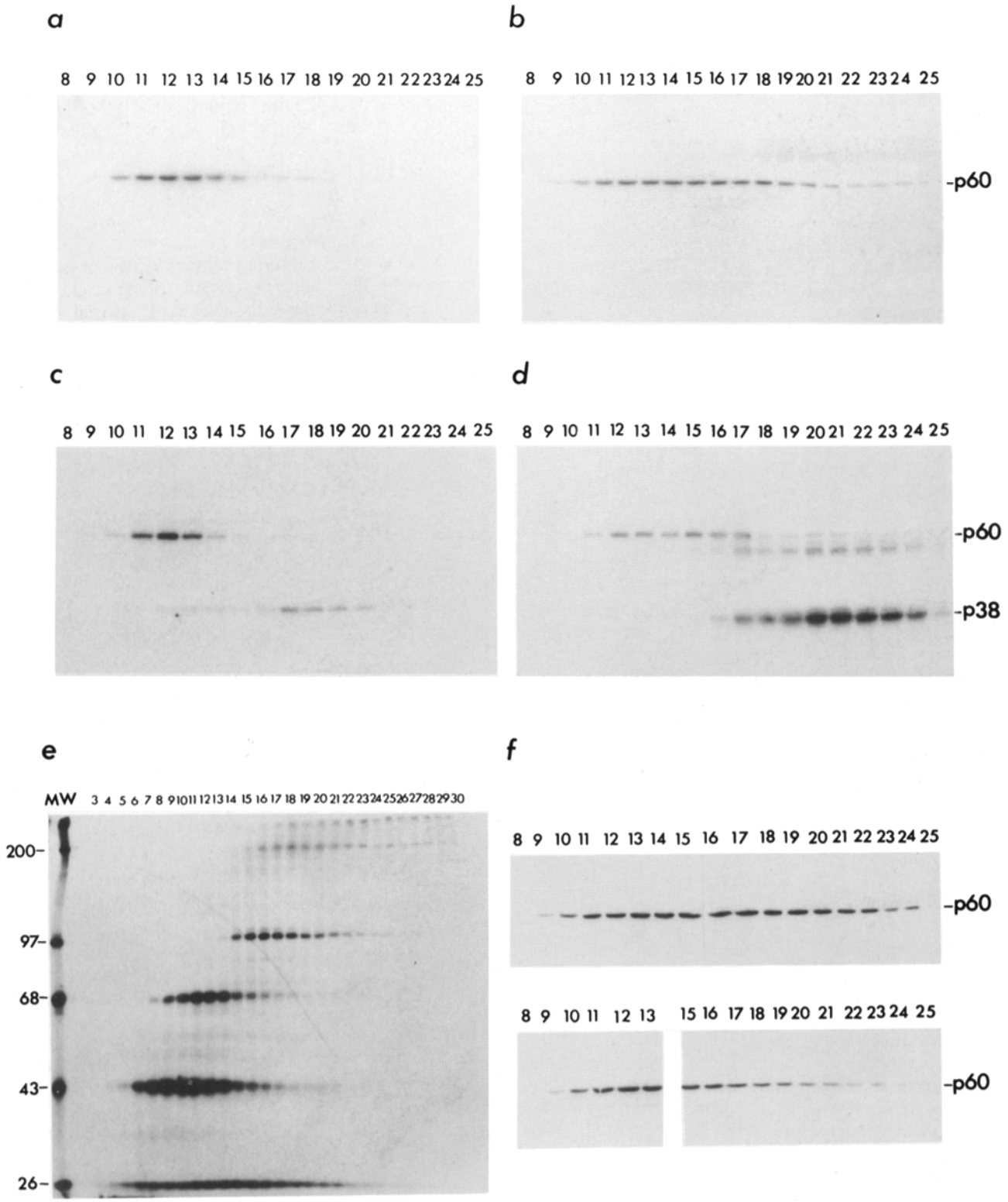

Figure 3. Glycerol gradient sedimentation profile of $p60^{c\text{-}src}$ from PM and CGM lysates. 10–25% gradient was separated into 30 fractions of 150 μl each and 75 μl was used for Western blot analysis with mAb 327. PM and CGM in *a* and *b*, respectively. The other half was immunoprecipitated with 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 ^{14}C-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 p60$^{c\text{-}src}$ proteolytic products present in the complex with p38 relative to the intact p60$^{c\text{-}src}$ 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 p60$^{c\text{-}src}$ sedimentation profile, analyzed by Western blot, collapsed to a narrower peak shifted to the left (Figs. 3 *f* and 4 *C*). Thus the stability of the complex is likely to be dependent on disulphide bridges.

Phosphorylation of anti-p60$^{c\text{-}src}$ 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. 5 *a*, 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. 5 *b*, 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 p60$^{c\text{-}src}$ glycerol gradient sedimentation data and indicates that the complex could contain either one or two p38 molecules directly linked via disulphide bonds to p60$^{c\text{-}src}$. The relatively low level of p60 phosphorylation detected in Fig. 5 *b* suggests that in vitro autophosphorylation in the complex might be suppressed. The 65-kD band, which was more prominent under nonreducing conditions, (Fig. 5 *a*, lanes *2* and *3*) was also analyzed in the presence of DTT and found to comigrate with p60$^{c\text{-}src}$ (Fig. 5 *b*, 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. 5 *a*, lane *3*, and 6 *a*, lane *2*). Similarly, overnight exposure of isolated chromaffin granules to 1 mM 2-mercaptoethanol did not affect the detection of the complex (Fig. 6 *b*, lane *2*). Conversely, 1 h exposure of CGM RIPA lysates to 1 mM 2-mercaptoethanol promptly caused disruption of p38/p60 complex (Fig. 6 *c*, 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. 5 *a*, lane *3*).

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

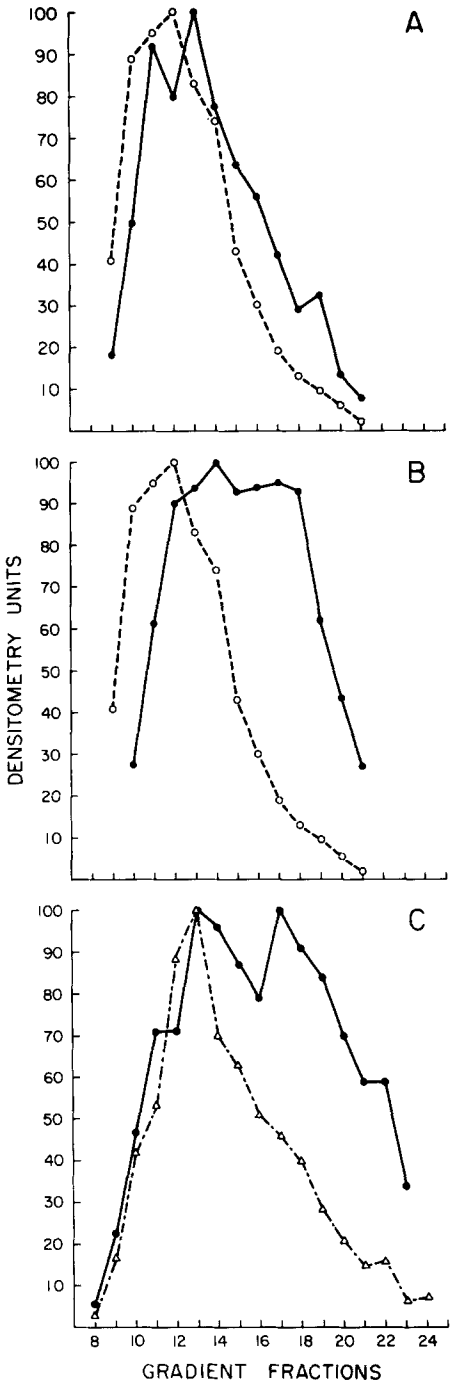

Figure 4. Quantitation by densitometry of the distribution of p60$^{c\text{-}src}$ in glycerol gradients analyzed by Western blotting with mAb 327. (*A*) The data displayed by ○ show the profile of p60$^{c\text{-}src}$ from CEF overexpressing the c-*src* gene from a recombinant retroviral vector (Levy et al., 1986). Although a small fraction of p60$^{c\text{-}src}$ 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 p60$^{c\text{-}src}$ from PM of adrenal medullae. (*B*) ○, control as in *A*. ●, sedimentation profile of p60$^{c\text{-}src}$ 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 p60$^{c\text{-}src}$ from CGM. △, the same lysate separated on the gradient in the presence of DTT. The direction of sedimentation is left to right.

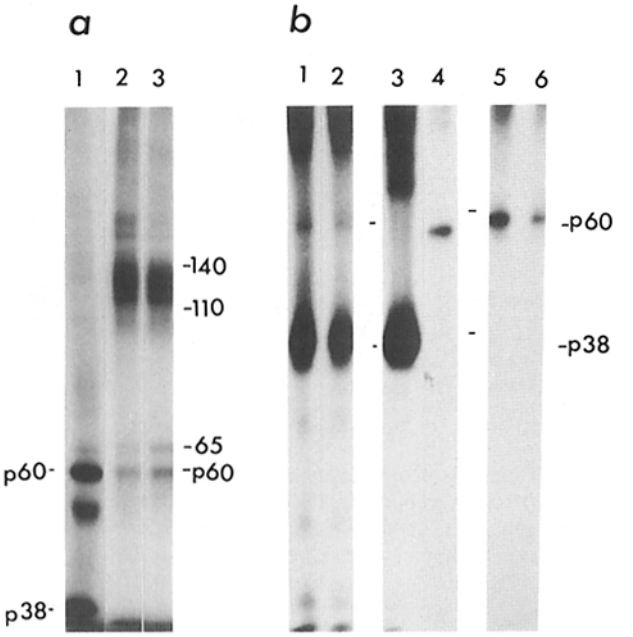

Figure 5. Nonreducing SDS-PAGE analysis of p38/p60 complex. (*a*) Immunoprecipitates with mAb 327 of CGM RIPA lysates were phosphorylated in a kinase assay, boiled in sample buffer with or without reducing agents (lanes *1* and *2*, respectively), and analyzed by 7.5% SDS-PAGE. Lane *3* shows also nonreducing 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.

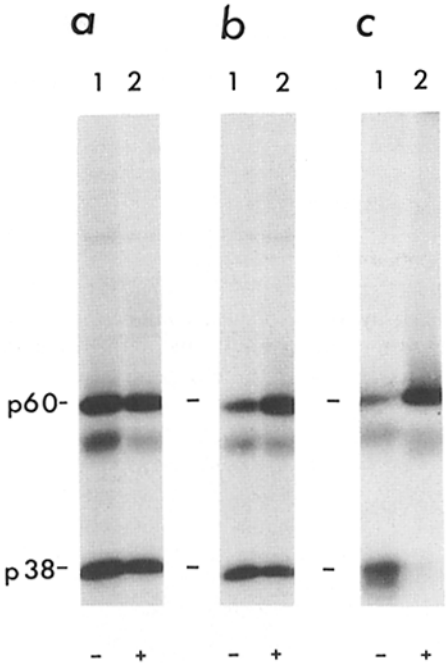

Figure 6. Effects of 1 mM 2-mercaptoethanol on the stability of the complex. (*a*) Adrenal medulla cell fractionation was performed in parallel without (lane *1*) or with (lane *2*) 2-mercaptoethanol until the isolation of chromaffin granules. CGM protein extracts were obtained by lysis in RIPA buffer 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.

enization buffers, no p38 phosphorylation could be detected in the immunoprecipitate, although $p60^{c\text{-}src}$ autophosphorylation occurred and was actually more prominent than in the control (Fig. 7 *a*, lanes *1* and *2*). Analysis by nonreducing SDS-PAGE did not show the broad 110–140-kD band in the CGM isolated in the presence of iodoacetamide (Fig. 7 *a*, lane *4*). Instead, a 65-kD band appeared. The same molecular mass band was previously detected, although it was less prominent in nonreducing gels of control samples (Fig. 5) and was also visible upon overexposure of autoradiographs from reducing gels. V-8 analysis of the 65-kD band from the iodoacetamide sample results in peptides indistinguishable from $p60^{c\text{-}src}$ (data not shown). The shift in molecular mass could be explained by an altered conformation of $p60^{c\text{-}src}$ stabilized by intrachain disulphide bonds or by the binding of a small protein, involving cysteine residues protected from alkylation. We tested the effect of iodoacetamide on the preformed complex. The results indicate that overnight exposure of chromaffin granules to 1 mM iodoacetamide (Fig. 7 *b*, lane *2*) had no major effects on the stability of the complex. However, 1-h exposure of CGM RIPA lysates to 1 mM iodoacetamide caused a partial disruption of the complex, as shown by the decrease of the p38 phosphorylation signal and the increase of p60 autophosphorylation (Fig. 7 *b*, lane *4*). This was confirmed by nonreducing SDS-PAGE analysis (data not shown).

In Vitro Kinase Activity of $p60^{c\text{-}src}$ in the Complex

To determine whether the specific activity of the $p60^{c\text{-}src}$ protein kinase is changed by complex formation, fractions from the glycerol gradient corresponding to either the monomer or complex form of $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$. Similar results were obtained when angiotensin was used as a substrate (data not shown). We conclude that there is no generalized increase in kinase activity of the complexed $p60^{c\text{-}src}$ relative to the monomer in vitro. In addition, the results indicate that p38 binding to $p60^{c\text{-}src}$ does not impair the ability of the enzyme to phosphorylate an exogenous substrate.

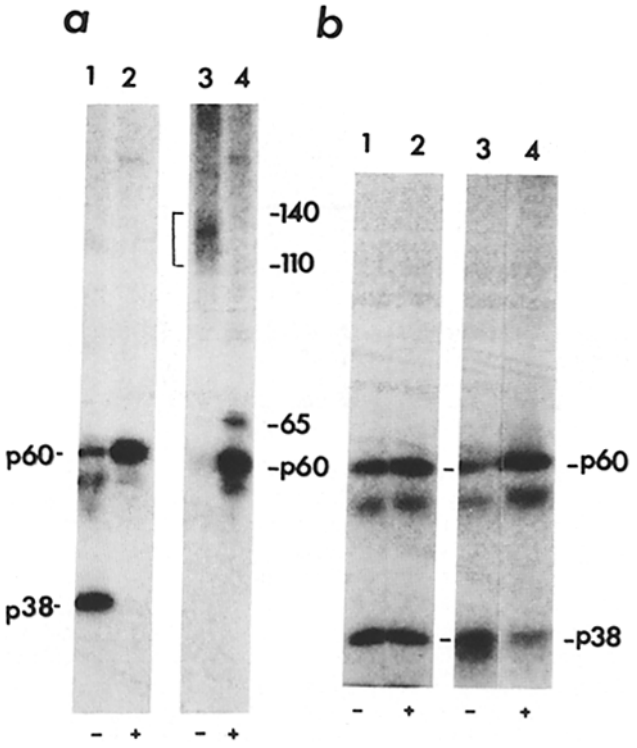

Figure 7. Effects of iodoacetamide on the stability of the complex. (*a*) Adrenal medulla cell fractionation was performed in parallel without (lanes *1* and *3*) or with (lanes *2* and *4*) 10 mM iodoacetamide. RIPA lysis of the CGM were also in the presence of iodoacetamide, immunoprecipitates were 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.

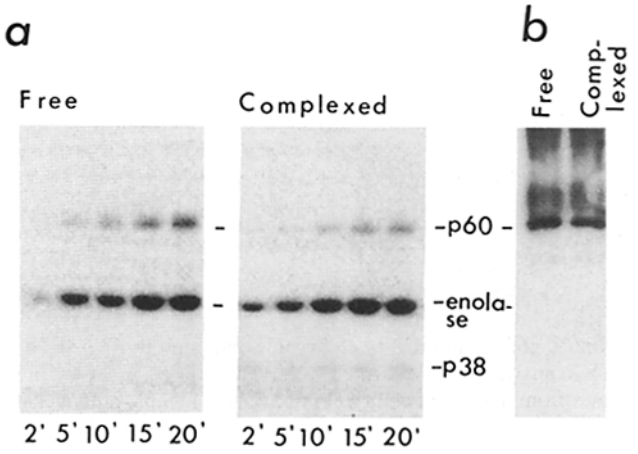

Figure 8. Comparison of the kinase activity of free vs. complexed $p60^{c\text{-}src}$. (*a*) Fractions numbered 10, 11, 12, 13 (free) and 17, 18, 19, 20 (complexed), obtained by glycerol gradient sedimentation, were pooled and immunoprecipitated with mAb 327. They were then divided into six tubes: one 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-^{32}P]ATP (3,000 Ci/mmole) 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 ^{125}I-labeled sheep anti-mouse Ig, and autoradiographed.

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 $p60^{c\text{-}src}$ (Bolen et al., 1984; Courtneidge, 1985). In agreement with the previous studies, we observed an increase in the kinase activity of the fraction of $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$. This effect can be observed by comparison of Fig. 3, *b* and *d* where the amount of $p60^{c\text{-}src}$ detected by Western blot is equivalent in the monomer and complexed form, yet the phosphorylation signal of the complexed $p60^{c\text{-}src}$ 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 (Fig. 6 *c*). Also, SDS-PAGE analysis of the phosphorylated complex excised from a nonreducing gel shows a higher ratio of p38 phosphorylation to p60 than in the unseparated monomer and complex mixture (compare Fig. 5 *b*, lanes *1* and 2, and Fig. 6 *a*). This phenomenon is detectable when a low ATP concentration (0.1 μM) is used in the kinase assay.

p38/p60 Complex Is Also Present in Brain

$p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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-

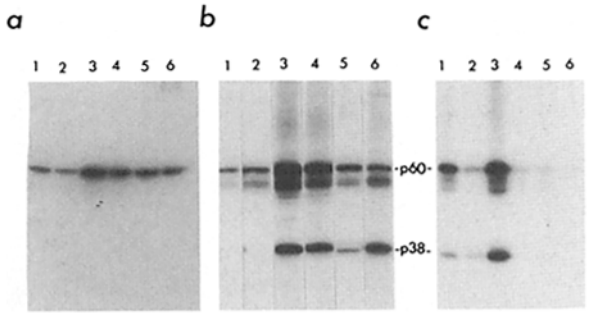

Figure 9. p60$^{c\text{-}src}$ and p38 in embryonic and adult brain. (*a*) Western blot analysis was performed with mAb 327 on 50 μg of protein lysates from day 18 rat embryo brain: (lane *1*) whole homogenate, (lane *2*) postnuclear supernatant, (lane *3*) subcellular fraction containing growth cones (collected at the 0.32–0.75 M sucrose interface), (lane *4*) heavier membrane fraction (collected at the 0.75–1.00 M sucrose interface). Chicken embryo brain crude membranes are shown in lane *5* and CGM in lane 6. (*b*) Kinase assay using 400 μg of protein lysates immunoprecipitated with mAb 327 and processed as described in the legend to Fig. 1. Lanes are labeled as in *a*. (*c*) Kinase assay of 200 μg of protein lysates immunoprecipitated with mAb 327 obtained by subcellular fractionation of adult chicken cerebella on a sucrose step grdient: (lane *1*) total homogenate, (lane *2*) 0.3–0.8 M interface, (lane *3*) 0.8–1.2 M interface, (lane *4*) 1.2–1.4 M interface, (lane *5*) 1.4–1.6 M interface, and (lane *6*) 1.6–2.0 M interface.

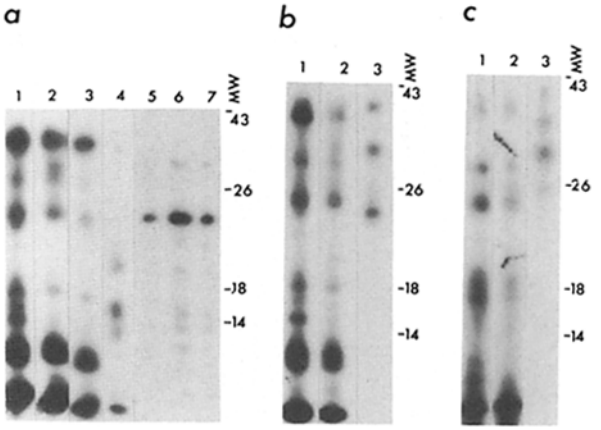

Figure 10. Peptide maps comparing various p38 proteins. Protein lysates were immunoprecipitated with mAb 327, in vitro phosphorylated in a kinase assay, and p60$^{c\text{-}src}$ and p38 were separated on a SDS-PAGE. Bands corresponding to both proteins were excised and subjected to partial V8 or chymotryptic cleavage (Cleveland et al., 1977; Collet et al., 1979). (*a*) V8 protease digestion products of p38 from different sources: (lane *1*) bovine CGM, (lane *2*) rat growth cones, (lane *3*) chicken embryo brains. V8 protease digestion products of p60$^{c\text{-}src}$: (lane *5*) bovine CGM, (lane *6*) rat growth cones, (lane *7*) chicken embryo brains. For comparison, V8 cleavage map is shown of calpactin I (also known as p36; lane *4*), obtained by in vivo labeling with ^{32}P-orthophosphate Rous sarcoma virus–transformed CEF, followed by immunoprecipitation with a polyclonal serum specific for calpactin I (Radke et al., 1984). (*b*) Lanes *1* and *2* are as in *a* but from a different gel. Lane *3* shows V8 cleavage products of synaptophysin obtained by in vitro phosphorylation of purified rat brain synaptic vesicles (kindly provided by Dr. Dennis Pang and Dr. Paul Greengard, The Rockefeller University, New York). Synaptophysin is exclusively phosphorylated on tyrosine under those conditions (Pang et al., 1988). (*c*) Chymotryptic digestion products of p38 from (lane *1*) bovine CGM, (lane *2*) rat growth cones, and (lane *3*) rat synaptophysin.

cal (Fig. 10 *a*, lanes *1* and *2*). Since previous developmental studies of p60$^{c\text{-}src}$ expression were done using chicken tissues (Fults et al., 1985; Cotton and Brugge, 1983; Sorge et al., 1984*a*,*b*), we decided to extend our analysis to chicken embryo brains at the stage when high levels of p60$^{c\text{-}src}$ are detected. Crude brain membranes were prepared by differential centrifugation from 14-d-old embryos. The kinase assay with mAb 327 showed a phosphoprotein migrating slightly faster than p38 (Fig. 9 *b*, lane *5*). This phosphoprotein was very similar to both bovine and rat p38 by V8 protease and chymotrypsin digestion (Fig. 10 *a*, lane *3* and data not shown).

We also looked for p38/p60 complex in chicken adult cerebella where p60$^{c\text{-}src}$ is still detectable, although the levels are decreased relative to the corresponding embryonal tissue. To identify a subcellular fraction enriched for p60$^{c\text{-}src}$, we separated the chicken cerebella homogenate into various subcellular fractions. The particulate portion of the homogenate was centrifuged through a sucrose step gradient and tested by kinase assay in the immune complex with mAb 327. The p38/p60 complex was detectable and enriched selectively at the 0.8–1.2 M sucrose interface (Fig. 9 *c*, lane *3*). We have not characterized this subcellular fraction but its sedimentation density is compatible with the presence of vesicles (Roda et al., 1980).

Discussion

In the present study we have shown that p60$^{c\text{-}src}$ is enriched in CGM isolated from bovine adrenal medullae and is complexed with a 38-kD protein. The enrichment of p60$^{c\text{-}src}$ 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$^{c\text{-}src}$ protein as detected by Western blot analysis, rather than an increase in the specific activity of the protein. Although the relative enrichment of p60$^{c\text{-}src}$ 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$^{c\text{-}src}$ is found in the granule membrane compartment. The 38-kD protein complexed with p60$^{c\text{-}src}$ in CGM is different from calpactin 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 calpactin 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ involve immunoprecipitation followed by a kinase assay or the sedimentation of $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$, 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 $p60^{c\text{-}src}$. It is possible that iodoacetamide disrupts the complex by altering protein structure and preventing disulphide bond formation. We hypothesize that p38 and $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ may have a regulatory role.

Is p38 a substrate of $p60^{c\text{-}src}$ in vivo? After labeling of primary adrenal cell cultures in vivo with ^{32}P-orthophosphate followed by immunoprecipitation with mAb 327 or antiphosphotyrosine antiserum, 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 $p60^{c\text{-}src}$ to chromaffin granules, and it could perform an analogous function in other tissues as well. Alternatively, p38 might be a modulatory protein of $p60^{c\text{-}src}$ activity. One protein that has been shown to stably associate with $p60^{c\text{-}src}$ is middle T antigen of polyoma virus which forms a complex with $p60^{c\text{-}src}$ in polyoma-transformed cells (Courtneidge and Smith, 1983, 1984). The association of middle T antigen increases the kinase activity of $p60^{c\text{-}src}$ (Bolen et al., 1984; Courtneidge, 1985). In contrast, the $p60^{c\text{-}src}$ complexed with p38 showed no increase in kinase activity under the conditions used in our experiments. It is possible that the substrate specificity of $p60^{c\text{-}src}$ is altered by complex formation or that the activation occurs transiently, under appropriate stimuli. Binding of p38 to $p60^{c\text{-}src}$ causes a decrease in the ability of $p60^{c\text{-}src}$, 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 $p60^{c\text{-}src}$, thereby impinging on the autophosphorylation site.

The subcellular localization of the complex in chromaffin granules, seems to suggest that the function of $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ 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 $p60^{c\text{-}src}$ with p38 seems to occur in a number of tissues where $p60^{c\text{-}src}$ is abundant and may play an essential role in $p60^{c\text{-}src}$ function in vivo.

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