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Association of p60\(^{c-src}\) with Endosomal Membranes in Mammalian Fibroblasts

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Abstract. We have examined the subcellular localization of p60\(^{c-src}\) in mammalian fibroblasts. Analysis of indirect immunofluorescence by three-dimensional optical sectioning microscopy revealed a granular cytoplasmic staining that co-localized with the microtubule organizing center. Immunofluorescence experiments with antibodies against a number of membrane markers demonstrated a striking co-localization between p60\(^{c-src}\) and the cation-dependent mannose-6-phosphate receptor (CI-MPR), a marker that identifies endosomes. Both p60\(^{c-src}\) and the CI-MPR were found to cluster at the spindle poles throughout mitosis. In addition, treatment of interphase and mitotic cells with brefeldin A resulted in a clustering of p60\(^{c-src}\) and CI-MPR at a peri-centriolar position. Biochemical fractionation of cellular membranes showed that a major proportion of p60\(^{c-src}\) co-enriched with endocytic membranes. Treatment of membranes containing HRP to alter their apparent density also altered the density of p60\(^{c-src}\)-containing membranes. Similar density shift experiments with total cellular membranes revealed that the majority of membrane-associated p60\(^{c-src}\) in the cell is associated with endosomes, while very little is associated with plasma membranes. These results support a role for p60\(^{c-src}\) in the regulation of endosomal membranes and protein trafficking.

p60\(^{c-src}\) is a member of a family of cytoplasmic tyrosine kinases that are associated with cellular membranes and are thought to be involved in signal transduction events underlying growth control (for review see Cooper, 1989). The normal function of p60\(^{c-src}\) is not clear, although studies of mutant forms of the protein (e.g., the viral transforming protein p60\(^{v-src}\)) have implicated p60\(^{c-src}\) in the control of cell growth and proliferation. On the other hand, p60\(^{c-src}\) is expressed at high levels in terminally differentiated cells such as platelets and neurons (Cotton and Brugge, 1983; Golden et al., 1986). In addition, p60\(^{c-src}\) is activated during mitosis in fibroblasts and thus may mediate certain mitotic events (Chackalaparampil and Shalloway, 1988; Morgan et al., 1989; Shenoy et al., 1989). Finally, a homozygous disruption of the c-src gene in mice is not lethal, but instead leads to defects in bone remodeling (probably as a result of defective osteoclast function) (Soriano et al., 1991).

Clues about p60\(^{c-src}\) function have been obtained from studies of the subcellular location of src proteins. Immunofluorescence and biochemical fractionation studies have suggested that both p60\(^{v-src}\) and p60\(^{c-src}\) are localized to perinuclear and plasma membranes, while transforming proteins have also been found in association with adhesion plaques (Courtneidge et al., 1980; Resh and Erikson, 1985; Rohrschneider, 1980). Immunofluorescence studies of p60\(^{c-src}\) have also revealed a punctate staining pattern that is dependent on membrane attachment domains in the amino-terminal region of the protein (Kaplan et al., 1990). This punctate pattern is characteristic of membrane vesicles, suggesting that p60\(^{c-src}\) is associated with cellular membranes distinct from the plasma membrane. Although these membranes remain uncharacterized, analysis of p60\(^{c-src}\) over expressed in 3T3 cells indicates a possible connection with endosomal membranes (David-Pfeuty and Nouvian-Dooghe, 1990). In addition, recent efforts to localize p60\(^{c-src}\) in differentiated PC12 cells and neuroendocrine cells have shown that a significant proportion of p60\(^{c-src}\) is associated with an endosomally derived population of vesicles (Linstedt et al., 1992; Grandori and Hanafusa, 1988).

To gain insight into the normal role of p60\(^{c-src}\), we have used high resolution immunofluorescence analysis and biochemical fractionation to characterize the nature of p60\(^{c-src}\)-containing membranes. Our results indicate that p60\(^{c-src}\) is mainly associated with endosomal membranes and is particularly enriched in a population of late endosomes.

Materials and Methods

Cells
The RTCS cell line was derived from a Rat-1 cell line infected with a retroviral construct expressing chicken p60\(^{c-src}\) (Morgan et al., 1989) and expresses ~2.5-fold more p60\(^{c-src}\) than the parental Rat-1 cell line (detected by Western blot; our own unpublished observations). In some experiments, we used cell lines derived from the spontaneous immortalization of mouse embryo fibroblasts isolated from mice heterozygous or homozygous for a disruption of the c-src gene (cells kindly provided by P. Soriano, Bay-
Antibodies

mAbs against p60src were kindly provided by Joan Brugge (University of Pennsylvania, Philadelphia, PA) (mAb327) and Sara Courtright (mAb2-17) (Lipschitz et al., 1983). Polyclonal rabbit sera against the cation-independent mannose 6-phosphate receptor (C1-MPR) were kindly provided by Peter Lobel (Rutgers University, New Brunswick, NJ) and Bill Brown (Cornell University, Ithaca, NY). mAbs against cismedial-Golgi markers (10E6 and 8B3) were also kindly provided by Bill Brown (Cornell University). Antibodies against BIP binding protein were obtained from David Bole (University of Michigan). mAb against β-tubulin (tub2.1) was purchased from Sigma Chemical Co. (St. Louis, MO). Texas red and fluorescein-labeled secondary anti-rabbit and anti-mouse antibodies were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY) and used at recommended dilutions.

Immunofluorescence

Cells were plated in six-well plates on glass coverslips 18-36 h before analysis. After specified treatments, cells were washed twice with PBS and then fixed for 20 min at 20°C in freshly made 3.7% paraformaldehyde/PBS (Polysciences Inc. Warrington, PA). Fixed endogenous antigens were washed twice with PBS and permeabilized with 0.1% Triton X-100 in PBS. Cells were rinsed and then blocked in PBS + 0.2% gelatin + 0.02% NaN₃. Primary antibodies were used at the following dilutions for 20 rain at 20°C in a humidified chamber: mAb 327 (1 mg/ml): 1/100; tub2.1: 1/200; CI-MPR; 1/200; mAb 2-17: 1/200; mAb 10E6/8B3: undiluted cell culture supernatant.Amphotericin B-tubulin (tub2.1) was treated with secondary antibodies at the appropriate dilutions. Endogenous levels of p60src in Rat-1 fibroblasts were detected with an unlabeled rabbit anti-mouse antibody (used at 1 µg/ml) followed by goat anti-rabbit antibody labeled with Texas red. Controls performed with only secondary and tertiary antibodies resulted in negligible signals. Coverslips were washed in PBS + 0.2% gelatin + 0.02% NaN₃, fixed again for 5 rain at 20°C, and then incubated finally in the presence of fluorescein-conjugated anti-mouse antibody and the mAb tub2.1. No tubulin staining was seen in the fluorescein channel, indicating that there was no interaction between fluorescein-labeled anti-mouse antibodies and mAb tub2.1.

Three-dimensional Optical Sectioning Microscopy and Image Processing

Data were recorded using a Peiltier-cooled charge-coupled device (CCD) camera (Photometrics Ltd., Tucson, AZ) mounted on an inverted microscope (Olympus Corp., Lake Success, NY). Images were collected with a 60x/1.4 oil immersion lens (Olympus Corp.); the effective CCD pixel size was 0.074 µm × 0.074 µm. Optical sections were recorded at 0.2 µm intervals. Multipie-wave length data were collected using a single dichroic mirror designed for DAPI, fluorescein, and Texas red emission. Three-dimensional data stacks were recorded, each was corrected for any photobleaching and lamp intensity variations which occurred during data collection (Paddy et al., 1990). Out-of-focus information was removed from each data stack by a three-dimensional, iterative deconvolution program with a non-negativity constraint (Agard et al., 1989). This method moves photons from their recorded positions (out-of-focus) to their points of origin without subtracting any of the information in the images. After the out-of-focus information was removed, projections representing the three-dimensional reconstruction of individual optical sections through the entire cell were generated. Data are presented both as projections of the entire set of optical sections, or as individual optical sections where noted. Some data are presented as unprocessed images recorded by the CCD camera.

Membrane Isolation and Fractionation

RTCS cells were plated 18-36 h before harvesting and allowed to reach a final confluence of 75-85%. Cells were loaded with HRP (Sigma Chemical Co.) (7.5-10 mg/ml in DME H-16, 10% FCS) at 37°C for 30 min (sufficient to saturate the endocytic pathway in these cells). Cells were chilled on ice, washed 4 times in ice-cold PBS and then scraped into homogenization buffer (20 mM Heps, pH 7.4, 150 mM KCl, 2 mM MgCl₂, 10 mM EDTA, 1 mM PMSF, 1 µg/ml leupeptin, 10 µg/ml pepstatin, 0.1 mg/ml soybean trypsin inhibitor, 1 U/ml aprotinin, 0.25 M sucrose). Cells were homogenized in a 2-ml dounce (Wheaton Inds., Millville, NJ) with an A pestle for 30-40 strokes. After homogenization, nuclear membranes were pelleted for 10 min at 2,000 g in an SS-34 rotor (Sorvall, Du Pont, Wilmington, DE) at 4°C. The postnuclear supernatant (SN) was collected and loaded over a 62% sucrose cushion and spun at 100,000 g in an SW50.1 rotor for 35 minutes at 4°C. Membranes at the interface were resuspended in the sucrose cushion (adjusted to 45% sucrose) and placed at the bottom of a sucrose step gradient consisting of 45, 52, and 18% steps. The gradient was centrifuged at 35,000 rpm in an SW50.1 rotor for 60 min at 4°C. The 18/32% interface was collected as a 23-gauge needle and a 2.5-ml syringe. The remaining fractions were collected from the top of the gradient with a Pasteur pipette and analyzed for p60src and membrane markers.

Under these homogenization conditions (30-40 strokes) 30-40% of HRP activity was left in the nuclear pellet and plasma membranes were mainly observed in the 45% sucrose fraction. Harsher homogenization conditions (80 strokes) left <1% of the total HRP activity in the nuclear pellet and the majority of plasma membranes were observed in the 32% and interface (18/32%) fractions on sucrose gradients.

To analyze the 18/32% sucrose interface on percoll gradients, the fraction was mixed into a 27% percoll solution (8.5% sucrose) and centrifuged over a 62% sucrose cushion in a 50T rotor at 16,000 rpm for 60 min at 4°C. Fractions were collected from the bottom of the tube and assayed for membrane markers and p60src by immunoblotting.

Membrane markers were assayed as follows. The plasma membrane marker alkaline diaphophoesterase I (ADPE I) was measured with thymine-5′-monophosphate-p-nitrophenyl ester (Sigma Chemical Co.) and assayed using a Beckman fluorometer at excitation 355 nM and emission 455 nM (Poole et al., 1983). The lysosomal marker β-hexosaminidase was measured with the fluorometric substrate 4-methyl umbelliferyl-β-D-glucosaminide (Sigma Chemical Co.) and samples were read on a Beckman fluorometer at excitation 350 nM and emission 450 nM (ref). The Golgi marker galactosyl transferase was measured by incorporation of UDP-[3H]-galactose (Amersham Corp., Arlington Heights, IL) into BSA (Aoki et al., 1985). HRP activity was measured using o-dianisidine (Sigma Chemical Co.) prepared at 0.1 µM/ml in PBS, pH 5.0, 0.1% Triton X-100. Activity was measured at OD₅00 at several time points (or at several volumes of extract) to ensure linearity of the assay.

Density Shift and DAB Cytochemistry

HRP-containing membranes from the 18/32% interface or from the P100 fraction (isolated under relatively harsh homogenization conditions; see above) were treated with DAB in homogenization buffer to a final concentration of 6 µg/ml. 10 µl of 30% H₂O₂ was added and the mixture incubated in the dark at 0°C for 10 min. After treatment, membranes were loaded on another sucrose gradient or a percoll gradient as described above, centrifuged, and analyzed for p60src and membrane markers. In control experiments no density shift was observed if DAB or H₂O₂ were added separately.

To inhibit HRP entry in some experiments, cells were chilled on ice and cold media containing HRP was incubated with the cells at 0°C for 20 min. Half of the cells were then harvested and membranes harvested as above. DAB cytochemistry was carried out and fractions from percoll gradients were analyzed for p60src and membrane markers.

1. Abbreviations used in this paper: ADPE I, alkaline diaphophoesterase I; BFA, brefeldin A; CCD, charge-coupled device; C1-MPR, cation-independent mannose 6-phosphate receptor; MTOC, microtubule organizing center; TGN, trans-Golgi network.
Monitoring of plasma membranes by surface iodination was performed with cells loaded for 30 min at 37°C with HRP. Cells were chilled on ice, washed 4 times with ice cold PBS and then dislodged from the plate using PBS + 1 mM EDTA. Cells were extensively washed in ice cold PBS and then labeled with AutoSIP (Pierce Chemical Co. Rockford, IL) labeled with iodobeads (Thompson et al., 1987). Free 125I counts were washed out of the cells using ice cold PBS. Membranes were isolated and DAB cytochemistry was performed and analyzed as above.

**Immunoprecipitation and Immunoblotting**

For immunoprecipitating p60<sup>-src</sup>, fractions were diluted two times with homogenization buffer and adjusted to 1% Triton X-100. mAb 327 was added and incubated at 4°C for 1-4 h. Protein A-Sepharose conjugated to rabbit anti-mouse antibody was added (50 µl/fraction of 50% slurry) and incubated at 4°C for 1-2 h. After one wash with homogenization buffer + 1% Triton X-100, protein A-Sepharose beads were boiled in loading buffer, and supernatants were loaded onto 10% PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose on an electroblot apparatus (Bio-Rad Laboratories, Cambridge, MA), and blots were blocked in 2% BSA for 1 h at 20°C. Blots were incubated in mAb327 (1 µg/ml) at 4°C for 18-24 h. Secondary 125I-labeled goat anti-mouse IgG (New England Nuclear, Boston, MA) was incubated for 1-4 h at 4°C and the blot washed and autoradiography carried out at ~70°C. Bands were cut from the blot and counted to measure relative p60<sup>-src</sup> signals. In some experiments, HRP-linked goat anti-mouse IgG (Amersham Corp.) was detected with a chemiluminescence procedure as described for the Amersham ECL detection system. To recognize antibodies against the CI-MPR, HRP-linked goat anti-rabbit antibodies (Amersham Corp.) were used in the chemiluminescence protocol.

**Results**

**Co-staining of p60<sup>-src</sup> with Late Endosomes at the Microtubule Organizing Center**

Rat-1 fibroblasts over-expressing chicken p60<sup>-src</sup> (RTCS cell line) were analyzed by indirect immunofluorescence with a mAb against p60<sup>-src</sup> (mAb 327). To enhance resolution, immunofluorescence was recorded with an optical sectioning mAb against p60<sup>-src</sup> (mAb 327). To enhance resolution, induction of the baculovirus expression system (Morgan et al., 1991) (data not shown). We therefore conclude that myristylation is necessary but not sufficient to allow MTOC localization of p60<sup>-src</sup>, supporting the notion that there are other domains of p60<sup>-src</sup> that contain information important for localization (Kaplan et al., 1990).

Membrane organelles involved in various secretory and endocytic processes have been localized to the MTOC. To identify candidate membranes that contain p60<sup>-src</sup>, co-localization studies were performed with a number of antibodies against markers of specific membrane compartments. The Golgi compartment was identified with several markers, including fluorescein-conjugated wheat germ agglutinin and two mAbs, 10E6 and BB3, specific for cis/medial–Golgi elements (Wood et al., 1991). These markers stained a perinuclear region of the cell that was distinct from p60<sup>-src</sup>, suggesting that p60<sup>-src</sup> is not found in cis/medial–Golgi membranes (Fig. 2 C). Similarly, staining of the ER with antibodies against BiP (Bole et al., 1989) and staining of lysosomes with antibodies against lgp120 (Lewis et al., 1985) revealed that these two membrane compartments are clearly distinct from p60<sup>-src</sup> at the MTOC (data not shown).

In contrast, staining with antibodies to the CI-MPR, a marker of endosomes and trans-Golgi network (TGN), revealed a striking co-localization with p60<sup>-src</sup> at the MTOC (Fig. 2, D and E). While all endosomal membranes are believed to contain the CI-MPR, the CI-MPR-positive endosomes located at the MTOC represent vesicles at a late stage in the endocytic pathway (late endosomes or pre-lysosomes) (Messner and Kreis, 1989). CI-MPR-positive vesicles also require intact microtubules for their localization, unlike early endosomes (K. Kaplan, unpublished observations) (Matteoni et al., 1987). Thus, our results suggest that p60<sup>-src</sup> is associated with membranes at the MTOC that represent a late stage in the endocytic pathway. While no di-
rect attempts were made to distinguish CI-MPR-positive endosomes from CI-MPR-positive TGN, we observed that p60\(^{-}\) did not exhibit the tubular staining pattern characteristic of the TGN (data not shown).

**P60\(^{-}\) and CI-MPR Co-localize after Treatment with BFA**

To further analyze p60\(^{-}\)-containing membranes and to distinguish these membranes from cis/medial-Golgi network, we treated cells with brefeldin A (BFA). BFA disrupts the normal distribution of cis/medial-Golgi membranes, causing retrograde transport to the ER. This results in a reticular staining pattern when the Golgi apparatus is visualized by immunofluorescence (Lippincott-Schwartz et al., 1989, 1990). In contrast, BFA treatment does not result in the transport of CI-MPR-containing membranes into the ER (Chege and Pfeffer, 1990; Lippincott-Schwartz et al., 1991; Wood et al., 1991). If p60\(^{-}\) is associated with CI-MPR-containing late endosomes, then the behavior of p60\(^{-}\) and CI-MPR after BFA treatment should be similar, and yet distinct from cis/medial-Golgi membranes.

Consistent with previous findings, we found that after BFA treatment the Golgi marker 10E6 exhibited a punctate/reticular staining pattern characteristic of ER staining in these cells (Fig. 3 A). We also observed that CI-MPR-positive membranes do not enter the ER after BFA treatment. After BFA treatment, both p60\(^{-}\) and CI-MPR (Fig. 3, B and C) formed a tight cluster at the centrosome in a staining pattern that is distinct from cis/medial-Golgi membranes. This clustering of membranes containing p60\(^{-}\) and CI-MPR at the centrosome is less diffuse than the staining pattern at the MTOC in untreated cells. The clustering occurs after 30 min of BFA treatment, is reversible, and is dependent on intact microtubules, since pretreatment with nocodazole abolishes the effect (data not shown). In untreated populations of fibroblasts, some cells (5-10\%) exhibit a similar clustering of p60\(^{-}\) and CI-MPR staining at the centrosome, suggesting that the clustering of endosomes in this region can occur in the absence of BFA. These results further support the notion that p60\(^{-}\) and the CI-MPR are in a similar cellular compartment that is distinct from the cis/medial-Golgi apparatus.

**Co-localization of p60\(^{-}\) and CI-MPR at the Spindle Poles during Mitosis**

The activation of p60\(^{-}\) during mitosis suggests that it may play a role in mitosis. Therefore we also examined the localization of p60\(^{-}\) in mitotic cells (identified by the state of their chromatin after Hoechst staining; see Materials and Methods). During mitosis, p60\(^{-}\) was observed to cluster at the dividing centrosomes (Fig. 4 A; arrows). Co-staining with anti-CI-MPR clearly demonstrated that the CI-MPR is clustered at the same peri-centriolar position as p60\(^{-}\) (Fig. 4 B; arrows). Reconstructive analysis of three-dimensional optical sections confirmed that p60\(^{-}\) staining corresponded to the position of migrating centrioles as determined by tubulin staining (data not shown). Analysis of additional stages of mitosis showed that the peri-centriolar populations of p60\(^{-}\) and CI-MPR were present throughout mitosis and appear positioned to form membrane organelles at the MTOC after telophase.

Over-expression of p60\(^{-}\) did not affect staining, as similar patterns were apparent when endogenous levels of p60\(^{-}\) were examined in Rat-1 cells (Fig. 4, D and E). The mitotic localization of both CI-MPR and p60\(^{-}\) is dependent on a properly formed spindle, since cells treated with nocodazole no longer show pericentriolar staining of p60\(^{-}\) or CI-MPR (data not shown). In addition, staining of cells with antibodies specific for the Golgi apparatus revealed that Golgi membranes became vesicularized and were scattered throughout the cell during mitosis as previously reported for HeLa cells (Luqcoq and Warren, 1987; Moskalenko and Thyberg, 1990). This contrast between Golgi staining and p60\(^{-}\)/CI-MPR staining during mitosis provides additional evidence that p60\(^{-}\) associates with endosomal membranes and not with cis/medial-Golgi elements.

**Biochemical Fractionation Reveals that p60\(^{-}\) Co-enriches with Endosomal Membranes**

We next examined the association of p60\(^{-}\) with endosomal membranes by standard cell fractionation techniques. RTCS cells were allowed to internalize the fluid phase marker HRP to label the endocytic compartment. Cellular membranes isolated after a 100,000 g spin (P100 pellet) were placed at the bottom of a sucrose step gradient consisting of 45, 32, and 18\% steps. Membranes were allowed to float to equilibrium during centrifugation. We determined that the endocytic marker HRP and thus endocytic membranes from these cells were enriched at the 18/32\% interphase of the gradient (data not shown). We analyzed the fractions from the gradient by immunoblotting to identify p60\(^{-}\) and observed a major peak at the 18/32\% interface (Fig. 5 A). Based on immunoblots with \(^{125}\)I-labeled antibody, we estimate that 65\% of the total p60\(^{-}\) on the gradient was at the 18/32\% interface (Fig. 5 B). A similar pattern of enrichment was observed with cells expressing endogenous levels of p60\(^{-}\) (data not shown). The density at the 18/32\% interface is characteristic of endosomal membranes and is therefore consistent with the enrichment of p60\(^{-}\) with endocytic vesicles. Analysis of markers for membrane compartments demonstrated that fractions enriched for p60\(^{-}\) are distinct from lysosomal (data not shown), Golgi apparatus, and plasma membrane markers (Fig. 5, C–E), although a small

![Figure 1](image-url)
Figure 2. Myristylation of p60<sup>++</sup> is required for localization at the MTOC and for co-staining with CI-MPR. 3T3 fibroblasts expressing a nonmyristylated mutant form of chicken p60<sup>++</sup> stained with mAb 327 (A) and anti-sera against CI-MPR (B) are presented as single unprocessed images. RTCS fibroblasts were stained with mAb 10E6 against cis medial-Golgi and a single unprocessed image is presented (C). RTCS fibroblasts stained with mAb 327 (D) and anti-sera against CI-MPR (E) are presented as projections. Bar, 10 μm.
Figure 3. Brefeldin A treatment causes p60\textsuperscript{cis} and CI-MPR membranes to cluster at the centrosome. RTCS fibroblasts were treated with 1\,\mu\text{g/ml} brefeldin A for 30 min at 37°C and then processed for immunofluorescence. A single unprocessed image of staining with mAb 10E6 against \textit{cis}/\textit{medial}-Golgi is presented (A), while projections of multiple optical sections are presented for staining with mAb 327 against p60\textsuperscript{c} (B) and anti-sera against CI-MPR (C). Arrows indicate clustered p60\textsuperscript{c} and CI-MPR staining at the centrosome. Bar, 10\,\mu\text{m}.

amount of plasma membrane marker was found at the 18/32\% interface (see Materials and Methods). P60\textsuperscript{c} was sometimes observed in the 45 and 32\% fractions. To address whether this material was membrane-associated, each fraction from the sucrose step gradient was diluted to a final concentration of 8.5\% sucrose, pelleted at 100,000\,g and the pellet analyzed for p60\textsuperscript{c} by immunoblotting. Approximately 90\% of the p60\textsuperscript{c} in these frac-

Figure 4. Co-staining of p60\textsuperscript{c} and CI-MPR at the spindle poles of mitotic cells. A–C represent projections of the same RTCS fibroblast prophase cell stained with mAb 327 against p60\textsuperscript{c} (A), anti-sera against CI-MPR (B) and Hoechst stain (C) to reveal the chromatin. Projections were obtained of Rat-1 fibroblasts stained with mAb 327 against p60\textsuperscript{c} plus a sandwich antibody (see legend to Fig. 1; D) and with Hoechst stain to reveal the chromatin (E). Arrows indicate peri-centriolar staining in mitotic cells. Bar, 10\,\mu\text{m}.
Figure 5. Enrichment of p60\(^{\text{c-src}}\) and endosomal membranes on sucrose step gradients. RTCS fibroblasts were incubated with HRP for 30 min at 37\(^\circ\)C and cell membranes were isolated and loaded on the bottom of a sucrose step gradient (see Materials and Methods). After centrifugation, fractions were analyzed by immunoblot with mAb 327 detected by \(^{125}\)I labeled goat anti-mouse antibodies (A). Bands were cut from the blot and counted (B). Fractions were also analyzed for HRP activity (endosomal marker) (C), ADPE I activity (plasma membrane marker) (D), and galactosyl transferase activity (cis/medial-Golgi marker) (E).

Shift in Density of Endocytic Membranes Alters Membrane-Bound p60\(^{\text{c-src}}\) Density

To determine if p60\(^{\text{c-src}}\) is directly associated with endocytic vesicles inside the cell we specifically altered the density of endocytic membranes as described previously (Cortney et al., 1984; Stoorvogel et al., 1991). Cells were allowed to internalize HRP to saturate the endocytic compartment (see Materials and Methods). Cellular membranes were isolated and sedimented on a sucrose step gradient as above. The 18/32 % interface was isolated and split equally. Half of the interface membranes were treated with DAB and hydrogen peroxide (H\(_2\)O\(_2\)), a substrate and catalyst for HRP. The modification of DAB by HRP in endocytic vesicles results in an increase in vesicle density. Each set of membranes was analyzed on separate sucrose step gradients for the presence of p60\(^{\text{c-src}}\) and HRP activity. As expected, DAB and H\(_2\)O\(_2\) treatment resulted in a shift of the endocytic marker HRP to the 45 % fraction (Fig. 7 B). Treatment of membranes with DAB and H\(_2\)O\(_2\) also resulted in a shift of membranes containing p60\(^{\text{c-src}}\) (Fig. 7 A) and CI-MPR (data not shown) to the 45 % fraction. Other membrane markers present in the 18/32 % interface did not undergo a shift in density to the 45 % fraction after treatment with DAB (Fig. 7, C and D). The variation in the distribution of the Golgi marker galactosyl transferase (Fig. 7 D) was found to be dependent on homogenization conditions rather than DAB treatment, suggesting that the DAB-induced density shift was specific for...
endosomal membranes. To demonstrate dependence of the density shift on the HRP reaction, samples treated only with DAB (or only with H₂O₂, data not shown) exhibited no shift in the apparent density of p60<sup>pro</sup>-associated membranes (Fig. 7 A, left).

**Association of the Majority of p60<sup>pro</sup> with Endosomal Membranes**

All of the p60<sup>pro</sup> isolated from the 18/32% fraction shifted density in these experiments, suggesting that all of the p60<sup>pro</sup> in this fraction is associated with membranes accessible to the endocytic marker HRP. To determine the amount of p60<sup>pro</sup> in the entire cell that is associated with endosomal membranes, we performed density shift experiments on the entire population of cellular membranes isolated from a 100,000 g pellet. The proportion of p60<sup>pro</sup> in this pellet that undergoes a density shift should represent the proportion of membrane-bound p60<sup>pro</sup> associated with endocytic membranes. After treatment with DAB and H₂O₂, total cellular membranes were separated on a percoll gradient and fractions were analyzed to identify p60<sup>pro</sup> and relevant membrane markers. P60<sup>pro</sup> from untreated membranes paralleled the peak of endosomal membranes (HRP) in low density fractions (Fig. 8 A). Treatment of membranes with DAB and H₂O₂ shifted the density of all detectable p60<sup>pro</sup> and the endocytic marker HRP to higher density fractions (Fig. 8, B and C).

The shift in density of all detectable p60<sup>pro</sup> suggested that the majority of p60<sup>pro</sup> is associated with endosomal membranes and little or no p60<sup>pro</sup> is associated with...
To further address the specificity of the density shift, HRP was bound to the surface of cells at 0°C to prevent endocytosis and cell membranes were harvested and treated with DAB and H$_2$O$_2$. No detectable density shift of p60$^{src}$ was observed (data not shown) despite high levels of HRP activity in the membrane pellet. Thus, only HRP internalized into the endocytic pathway is able to shift the density of p60$^{src}$-containing membranes.

It was also possible that there was a preferential solubilization of plasma membrane-associated p60$^{src}$ during preparation of cellular membranes. The 10–20% of total p60$^{src}$ that we detected in the soluble S100 fraction may therefore represent a plasma membrane-associated fraction of p60$^{src}$. To address this possibility, cell extracts were treated with DTSSP (as described above) to reduce the amount of p60$^{src}$ in the S100 fraction. P60$^{src}$ was fractionated on Percoll gradients and did not cosediment with plasma membranes (data not shown). It is therefore unlikely that the population of soluble p60$^{src}$ came from the plasma membrane.

**Discussion**

We have found that the majority of membrane-associated p60$^{src}$ in mammalian fibroblasts is localized to a compartment that probably represents a population of endosomes. Our immunofluorescence experiments demonstrate that a significant proportion of p60$^{src}$ is found at the MTOC and co-localizes with Cl-MPR, a marker of late endosomes. Biochemical fractionation and density shift experiments indicate that the majority of p60$^{src}$ in the cell associates with endosomal membranes accessible to the endocytic marker HRP. These results raise the possibility that p60$^{src}$ plays a role in endosomal function.

Previous immunofluorescence and electron microscopic studies of p60$^{src}$ tend to support localization of the viral src protein at the plasma membrane (Rohrschneider, 1979; Willingham et al., 1979). Some src family members are thought to interact with cell surface proteins, and a putative plasma membrane receptor for p60$^{src}$ has been identified in cross-linking experiments (Resh and Ling, 1990). In contrast, our results suggest that very little p60$^{src}$ is associated with plasma membranes. Although we sometimes observed p60$^{src}$ staining at cell–cell contacts, we suspect that this does not represent plasma membrane association but rather is because of enrichment of membranes at bundles of microtubules (Fig. 1). Dependence on intact microtubules for this localization further supports the notion that p60$^{src}$ is microtubule-associated at cell–cell contacts. The discrepancy between previous localization studies and our results may arise in part from differences between the transforming protein p60$^{src}$ and the nontransforming protein p60$^{src}$.

Our results also differ from previous studies that have used biochemical fractionation to localize src proteins. These studies demonstrate an enrichment of both p60$^{src}$ and p60$^{src}$ in plasma membranes (Courtneidge et al., 1980; Resh and Erikson, 1985). Fractionation techniques used in these studies did not differentiate between plasma membranes and endosomal membranes, which have similar densities and are likely to cosediment. We have used variable conditions during homogenization to alter the sedimentation of plasma membranes, allowing them to be separated from endosomes...
Figure 8. DAB-induced density shift of p60<sup>src</sup> from total cellular membranes. RTCS fibroblasts were incubated with HRP and total cellular membranes were isolated. Membranes were treated with DAB alone (A), or DAB/H<sub>2</sub>O<sub>2</sub> (B) and then run on percoll gradients. Fractions were collected as in Fig. 6. P60<sup>src</sup> was detected by immunoprecipitation and immunoblotting with mAb 327. Fractions were also analyzed for HRP activity to detect endosomal membranes (■) and a gamma counter was used to detect the presence of iodinated plasma membrane proteins (△). Note that plasma membranes do not behave as in Fig. 6 due to the absence of CaCl<sub>2</sub> (see legend to Fig. 6).

(see Materials and Methods). In addition, the specific shift in density of HRP-containing membranes convincingly separated endosomes from plasma membranes. Based on results from these fractionation experiments we have concluded that no detectable p60<sup>src</sup> is associated with plasma membranes. It remains possible that a very small proportion of p60<sup>src</sup> is associated with plasma membranes but is undetectable under our conditions.
Our results suggest that p60^src is associated with the microtubule cytoskeleton via endocytic vesicles, in contrast to previous localization and biochemical studies suggesting an association of p60^src with the actin cytoskeleton (Hamaguchi and Hanafusa, 1987; Henderson and Rohrschneider, 1987; Resh and Erickson, 1985; Rohrschneider, 1979, 1980). Analysis of optical sections near the bottom of cells over-expressing chicken p60^src showed only punctate, microtubule-associated staining and no localization to adhesion plaques. We found that p60^src co-localizes with a number of microtubule-related structures, including microtubule bundles at points of cell-cell contact, the microtubule organizing center, and a region associated with the spindle pole during mitosis (Figs. 1 and 4). In all cases this staining is dependent on intact microtubules. Ample evidence has demonstrated that endocytic membranes can be transported along microtubules (DeBrabander et al., 1988; Goergi et al., 1990; Matteoni and Kreis, 1987). It is therefore likely that co-localization of p60^src with various microtubule structures reflects the transport of endosomal membranes along cellular microtubules. It is even conceivable that p60^src may regulate microtubules or microtubule-associated motors involved in endosomal membrane transport.

The microtubule-dependent alteration of p60^src staining induced by BFA treatment provides additional evidence for p60^src transport along microtubules. BFA is known to cause microtubule-dependent retrograde transport of Golgi elements into the ER (Lippincott-Schwartz et al., 1989, 1990). We have shown that treatment of cells with BFA also affects the transport of endosomal membranes containing p60^src. BFA treatment results in the clustering of p60^src-containing membranes at the centrosome, which is distinct from the normally diffuse staining of p60^src at the MTOC (Fig. 3). Similar findings have been reported for the effect of BFA on a wide range of endocytic membranes in both rat and canine cells (Lippincott-Schwartz et al., 1991; Wood et al., 1991). While the mechanism of action of BFA remains unclear, the behavior of p60^src and CI-MPR after BFA treatment emphasizes the similar nature of the membrane compartment occupied by these proteins.

Studies of cells in early stages of mitosis show that p60^src and CI-MPR are found at the dividing centrosomes (Fig. 4). The presence of both CI-MPR and p60^src at the spindle poles during mitosis further suggests that both of these proteins reside in a similar membrane compartment that is distinct from cis/medial-Golgi membranes, which are scattered throughout the cell during mitosis (K. Kaplan, unpublished observations) (Lucocq and Warren, 1987; Moskalenko and Thyberg, 1990). Since p60^src is known to be activated during mitosis and phosphorylated by p34^cdc2 (Chackalaparampil and Shalloway, 1988; Morgan et al., 1989), it is possible that p60^src is mediating mitotic effects of p34^cdc2 at the pericentriolar region of the cell. Several processes associated with endosomal membranes are known to be regulated during mitosis. For example, it has been shown in vitro that early endosomal fusion events are inhibited by addition of active Xenopus p34^cdc2 (Tuomikoski et al., 1989; Woodman et al., 1992).

Work with secretory cells suggests that p60^src may be involved in regulating the function of specialized secretory vesicles. In addition to being enriched in secretory granules of chromaffin cells, p60^src is associated with a 38-kD protein that may be important in the function of these secretory organelles (Grandori and Hanafusa, 1988). These compartments may be analogous to the endosomal compartment in fibroblasts. The association of p60^src with endomysosomal-derived synaptic vesicles in PC-12 cells is consistent with this proposal (Johnston et al., 1989; Linstedt et al., 1992).

Endosomal membranes are dynamic structures involved in the trafficking of proteins throughout the cell and are known to be regulated at the level of both transport and fusion. In this regard it may be relevant to consider the phenotype of mice that are genetically null for the c-src gene through homologous recombination (Soriano et al., 1991). The defect in bone modeling (osteopetrosis) exhibited by these mice arises ultimately from an inability to dissolve bone tissue during development. The osteoclasts responsible for dissolving bone tissue are known to be highly specialized secretory cells that secrete lysosomal enzymes. In the absence of p60^src, these cells may be defective in regulating the trafficking of lysosomal proteins.

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