Discrete Primary Locations of a Tyrosine Protein Kinase and of Three Proteins That Contain Phosphotyrosine in Virally Transformed Chick Fibroblasts

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ABSTRACT We have studied the localization of three abundant cellular proteins which are substrates for tyrosine protein kinases in virally transformed chicken embryo fibroblasts. The primary location of each substrate is unaltered by transformation with Rous sarcoma virus (RSV). The tyrosine-phosphorylated species is localized with the nonphosphorylated species. Two of the proteins, of about 46,000 and 28,000 daltons, have a similar location. They are present in the high speed supernatant of cells homogenized in hypotonic buffer, and are soluble in nonionic detergent. The third protein, of about 39,000 daltons, is particulate when cells are homogenized in hypotonic buffer containing divalent cations, but ~30% is free in the high-speed supernatant when divalent cations are absent. This protein appears to be associated with the detergent-insoluble matrix when adherent cells are gently lysed in nonionic detergent in situ, but is soluble when the same cells are extracted with nonionic detergent in suspension. This suggests that none of the proteins are tightly associated with detergent-insoluble cytoskeletal structures, unlike the RSV transforming protein itself, which is the main tyrosine protein kinase known to be active in RSV-transformed cells.

The transforming proteins of many retroviruses have been extensively studied with regard to their possible enzymatic functions and their subcellular localization. Many of these proteins have associated tyrosine protein kinase activities (for references, see 1). In the case of the transforming protein of Rous sarcoma virus (RSV), pp60src, there is abundant genetic and biochemical evidence that the protein itself is a tyrosine protein kinase and that this activity is involved in cell transformation (2-5). The primary location of transforming proteins has been studied by cytological and biochemical techniques. For RSV, immunofluorescence (6-8) and immunoelectron microscopy (9) show that pp60src is cytoplasmic and that a significant fraction is at the inner face of the plasma membrane. In biochemical studies, hypotonic lysis and centrifugation show that pp60src is in the plasma membrane fraction, although some may be released proteolytically as a 47,000-52,000 dalton (47 kd-52 kd) polypeptide (10-13). It appears that the NH₂-terminal 8 kd is tightly linked to the membrane (13). This region of the protein has recently been found to contain covalently bound fatty acid which may contribute to its membrane location (Selton et al., Cell, In press). The transforming proteins of many other retroviruses appear to be associated with the plasma membrane (14).

An alternative biochemical approach for localizing proteins in the cell involves extraction with nonionic detergents. Extraction with polyoxyethylene glycol (PEG) cetyl alcohol (Brij 58) removes membrane lipids and ~10% of cellular protein (15). PEG p-t-octyl phenol (Triton X-100) extraction removes >50% of cellular protein but leaves a filamentous matrix, which includes the cytoskeleton, intact (16). This detergent-insoluble matrix of normal cells consists chiefly of bundles of actin microfilaments, called stress fibers, intermediate filaments, and microtubules, although the latter are solubilized unless Ca²⁺ ions are chelated. In RSV-transformed cells the microfilament bundles are disorganized (17-19) whereas the intermediate filaments and microtubules are less dramatically affected (20, 21). Transformation also causes changes in the cytoskeletal structures which link the cell to the substratum (focal adhesions) and in the precise inter-relationship between intermediate filaments and microtubules (22-24). Some of these alterations may be mediated by changes in abundance of cytoskeletal proteins (25), but many changes are rapid (26) and can occur in enucleated cells (27), and so, are more likely to be affected by posttranslational modifications such as phosphorylation (28).

pp60src has been found associated with the detergent insol-
able matrix (29). This result is not inconsistent with its membrane location since some membrane proteins are attached to cytoskeletal structures (30). Indeed, immunocytochemical studies show that pp60°c is concentrated near the membrane at the focal adhesions (23, 31) and at tight junctions between adjacent cells (9, 31). These structures contain cytoskeletal proteins, including vinculin, α-actinin, and actin (22, 23, 32, 33), as well as membrane proteins. Vinculin is phosphorylated at two specific tyrosine residues at elevated levels in RSV-transformed chicken cells, resulting in an increase in phosphotyrosine from 2% to 27% of the phosphoamino acids recovered from vinculin (28). Although only about 1–2% of the vinculin molecules contain phosphotyrosine in transformed cells, it has been argued that this phosphorylation could contribute to the redistribution of focal adhesions which accompanies transformation, and thus to the altered morphology of the transformed cell (28).

We have found that transformation by RSV induces the phosphorylation of other cell proteins on tyrosine (34–36). One protein, of ~39 kd (37–41), is also phosphorylated in certain cells when they are treated with epidermal growth factor (EGF) (42). In chicken cells, RSV induces phosphorylation of two other proteins of about 46 kd and 28 kd (34). It is not known which protein kinases are responsible for any of these phosphorylations in vivo, but it is known that pp60°c can phosphorylate the 39 kd protein in vitro (39).

In this paper we report cell fractionation experiments on uninfected and RSV-infected chicken cells, to generate (a) particulate and soluble fractions (b) cytoskeletal and noncytoskeletal fractions. Each fraction was examined for its content of the 46 kd, 39 kd, and 28 kd phosphoproteins and their presumptive precursors.

MATERIALS AND METHODS

Cells and Viruses

Chicken embryo cells (CECs) were prepared and infected with Schmidt-Ruppin strain RSV, subgroup A (M. Weber, University of Illinois, Urbana-Champaign, IL) as described (34). After several days, to allow transformation, the cells were reseeded at 10° cells per 35-mm dish or 2.5 X 10° per 50-mm dish. After 24 h they were labeled for 16–20 h at 41°C with 500 μCi 32p per ml of phosphate-free Dulbecco’s modified Eagle medium (DME) containing 4% calf serum, or with 100 μCi [3H]ethanolamine (Amersham Searle) per ml of DME containing 5% of the regular methionine concentration and 4% calf serum. 1 ml of labeling medium was used for 35-mm dishes and 2 ml for 50-mm dishes.

Sample Preparation for Two-dimensional Gel Electrophoresis

 Cultures were prepared for two-dimensional gel electrophoresis according to the procedure of Garrels (43). Briefly, 35-mm dishes were washed with cold Ca2+. Mg2+-free tris-(hydroxymethyl)aminomethane (Tris) buffered saline (TBS) and 100 μl of 0.1% NP-40 (Bheresda Research Laboratories, Inc., Gaithersburg, MD) in Staphylococcal nuclease solution (45) (called here, solution A) was added. The cells were scraped and transferred to a 1.5-ml Eppendorf tube. In rapid succession 10 μl DNAse I-RNAse A solution (43) (solution B) and 20 μl of 3% SDS, 10% 2-mercaptoethanol (solution C) were added with mixing and the sample lyophilized. The sample was dissolved in 100 μl of solution D for gel electrophoresis.

Cell Fractionation into Soluble and Particulate Fractions

Cell cultures (50-mm dishes) were washed twice with cold TBS and 0.7 ml of a solution containing 10 mM NaCl, 20 mM sodium phosphate pH 7.0, 1% Trasylol (Moby Chemical Corp., New York, NY), 14 mM 2-mercaptoethanol, 1 mM ethylene diaminetetraacetic acid (EDTA) was added. The cells were allowed to swell for 10 min at 4°C. They were scraped off the dish and transferred to a Teflon tissue grinder (Dual) size 20, Kontes Scientific Glassware, Vineland, Nl) and homogenized until phase contrast microscopy showed that all cells were broken. ~500 μl was transferred to a 3/4 X 1/2 inch cellulose nitrate tube (Beckman Instruments, Inc., Fullerton, CA) and centrifuged in a SW50.1 rotor at 40,000 rpm (150,000 g) for 30 min at 4°C. The supernatant and pellet (resuspended in 600 μl homogenization buffer) were prepared for two-dimensional gel electrophoresis by adding in rapid succession, with mixing, 25 μl of 30 mM MgCl2, 250 μl of solution A, 25 μl of solution B, 50 μl of solution C, and lyophilizing. The lyophilized material was dissolved in 250 μl of solution D shortly before electrophoresis. For cell fractionation in the presence of Mg2+ ions, the homogenization buffer contained 1 mM MgCl2 and no EDTA. In this case, further MgCl2 was not added before solution A.

Fractionation into Detergent Soluble and Insoluble Fractions

Cell cultures (35-mm dishes) were washed with TBS and either scraped from the dish and washed into a 1.5-ml Eppendorf tube or trypsinized with 0.5 ml TBS containing 0.5 mg trypsin (Sigma Chemical Co., St. Louis, MO). The trypsin was neutralized with 0.5 ml of cold TBS containing 1 mg of soybean trypsin inhibitor (Sigma Chemical Co.) per ml and the cells were washed twice by centrifugation in 1 ml of the same solution.

Cell pellets were extracted at 4°C by vortexing for 3–4 s with 100 μl of 100 mM KCl, 10 mM 1,4-piperazine diethanesulphonic acid pH 6.8, 0.5% NP-40, 300 mM sucrose, 1% Trasylol, 3 mM MgCl2. 32P-labeled cells were extracted in the same solution containing 1 mM ATP to stop 32p incorporation. After 3 min at 4°C the insoluble material was pelleted in a microcentrifuge for 30 s. The soluble material was withdrawn. The pellet was resuspended (5 s on Vortex mixer) in 500 μl extraction buffer, re-centrifuged, and then resuspended in 100 μl extraction buffer. To each fraction, 100 μl solution B, 20 μl solution C were added in rapid succession and the samples lyophilized and redissolved in 100 μl of solution D for gel electrophoresis.

Cells were also extracted on the dish. After washing with cold TBS, 100 μl of the above extraction solution were added and allowed to sit at 4°C for 3 min. The dish was then drained and washed once gently with 500 μl of extraction solution, and the detergent insoluble material scraped in 100 μl of extraction buffer. Both fractions were then prepared for gel electrophoresis.

Two-dimensional Gel Electrophoresis

Isoelectric focusing gels were prepared and run for 14,000 V h as described (34, 43) with 2% pH 6–8 range ampholytes or with 1.6% pH 6–8 plus 0.4% 8–10 range ampholytes. The latter conditions reproducibly allow the nonphosphorylated forms of the 39-kd and 28-kd proteins to enter the basic end of the gel. The second dimension gels contained 15% acrylamide and 0.087% bis-acrylamide (34). Gels containing 32P were incubated in alkali as described (34) and exposed with an intensifying screen. Gels containing 32P were impregnated with 2,5-diphenyloxazole. All autoradiographs were exposed at ~70°C with preexposed film.

Kinase Activity of pp60°c

Cells were lysed in radioimmune precipitation assay buffer containing 1 mM EDTA (44) or cell fractions were adjusted to the corresponding detergent and ionic conditions. Precipitation with tumor-bearing rabbit serum, immune complex kinase assays and gel electrophoresis of the labeled immunoglobulins were described (44).

RESULTS

Separation of Cells into Particulate and Soluble Fractions

Cells were broken by homogenization in hypotonic buffer and fractionated by centrifugation to obtain information on which proteins were present in membrane-bound organelles or other supramolecular structures. When RSV-transformed CECs were homogenized in the presence of EDTA and separated into fractions sedimenting at 130,000 g (particles larger than 40 S) and nonsedimentable material, the bulk of pp60°c, assayed by the immune complex kinase assay, was recovered in the high speed pellet. This fraction was also highly enriched for 5' nucleotidase, a plasma membrane marker (data not shown). When RSV-transformed CECs were homogenized and fractionated in the presence of Mg2+ ions, lower amounts of pp60°c kinase activity were recovered, but it was clearly par-
ticate. Since the location of pp60<sup>src</sup> has already been thoroughly investigated by others (10-12) we have not characterized these fractions further, and subsequently we fractionated the cells simply into "soluble" and "particulate" fractions by a single high speed spin, at 150,000 g.

For analysis of cellular phosphoproteins, RSV-transformed CECs were labeled with <sup>32</sup>P, homogenized in the presence of EDTA, and centrifuged at high speed. EDTA was included to inhibit unscheduled phosphorylation of proteins after lysis. The supernatant and pellet fractions were analyzed on two-dimensional gels and autoradiographed before (not shown) and after incubation in alkali (34; Fig. 1 A, B). The alkali treatment enhances the detection of phosphotyrosine-containing proteins (34, 45). By way of control, another dish of cells was prepared directly for two-dimensional gel electrophoresis according to our standard procedure (Materials and Methods), and electrophoresed in parallel with a mixture of the supernatant and pellet fractions (Fig. 1 C). No differences between the control gel and the mix were detected, suggesting that neither differential loss of proteins nor unscheduled phosphorylation or dephosphorylation had occurred during cell fractionation (not shown).

Three phosphoproteins were detected which we have previously found to contain phosphotyrosine and phosphoserine in RSV-transformed CECs but to be undetectable in alkali-treated two-dimensional gels of phosphoproteins of normal CECs (34; Fig. 1 C, arrowheads). These are the 46-kd, 39-kd, and 28-kd proteins (proteins α, β, and γ respectively of reference 34) which are our principal interest in this paper. The major portion of the alkali-resistant <sup>32</sup>P radioactivity in the 46-kd and 28-kd phosphoproteins was present in the high speed supernatant fraction (Fig. 1 A). The high speed pellet fraction contained some alkali-stable phosphoproteins including ~60% of the 39-kd phosphoprotein (Fig. 1 B) as well as many alkali-labile phosphoproteins (not shown).

Information regarding the subcellular location both of the transformation-dependent phosphoprotein and of their precursors was obtained from experiments with [35S]methionine-labeled cells. Since the addition of a phosphate group to a protein alters its isoelectric point, the phosphorylated and nonphosphorylated forms can be resolved by two-dimensional gel electrophoresis. Phosphoproteins found only in transformed cells can be identified as proteins which are unique to transformed cells and which comigrate with <sup>32</sup>P-labeled spots. Note, however that for the phosphoproteins of interest a single isoelectric species contains both phosphotyrosine and phosphoserine (34). These species are indicated in Fig. 2 (small arrowheads). Candidate nonphosphorylated forms of the 46-kd, 39-kd and 28-kd phosphoproteins have been identified by their position on two-dimensional gels. They are separated from the corresponding phosphoprotein, which is more acidic, by somewhere between 1 and 2 charges, the precise separation depending on the pK<sub>a</sub> of the phosphate group and the isoelectric point of the protein (34, 38, 39). These potential relationships have now been confirmed by peptide mapping and by the generation of specific antisera for the 46- and 39-kd proteins (Cooper and Hunter. Manuscript in preparation). These proteins are also marked on Fig. 2 (large arrowheads).

To determine the relative distribution of the two forms of each protein, [35S]methionine-labeled RSV-transformed CECs were homogenized in the presence of EDTA and separated into high speed supernatant and pellet fractions which were analyzed independently and in combination on two-dimensional gels (Fig. 2). The 46- and 28-kd phosphoproteins and their presumptive precursors were present predominantly in the supernatant fraction (Fig. 2 A, Table I). ~50% of the 39-kd phosphoprotein and 30% of its precursor were present in the supernatant.

Other investigators have found that a fraction of the 39-kd phosphoprotein is particulate, and have further localized this population to the plasma membrane fraction (S. Courtneidge, National Institute for Medical Research, London, K. Radke, P. Moss, and G. S. Martin, University of California, Berkeley; personal communications.). They found, however, that the most of the protein was particulate if the homogenization

![Figure 1 Fractionation of <sup>32</sup>P-labeled RSV-transformed CECs by homogenization in hypotonic buffer containing EDTA. (A, B) 12-h autoradiographs of alkali-treated two-dimensional gels (pH 6-8 range ampholytes) loaded with 10 μl of (A) supernatant or (B) pellet fractions. (C) 26-h autoradiograph of alkali-treated gel loaded with a mixture containing 5 μl of each fraction. Arrowheads: positions of 46-kd, 39-kd, and 28-kd phosphoproteins. These and all subsequent autoradiographs are shown with more acidic proteins on the left, and molecular mass decreasing down the gel (34).](image-url)
TABLE I

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<tr>
<th>Conditions</th>
<th>% particulate or insoluble</th>
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<td>46 kd</td>
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<td>Hypotonic lysis</td>
<td>+ EDTA</td>
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<td>Detergent lysis</td>
<td>+ Trypsinized cells</td>
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* [35S] methionine-labeled uninfected or RSV-transformed CECs were fractionated and analyzed by two-dimensional gels as described in the text. The regions of the gel corresponding to the nonphosphorylated forms of the 46-, 39-, and 28-kd proteins were excised, solubilized in NCS Tissue Solubilizer (Amersham-Searle) and radioactivity counted. The proportion of radioactivity recovered in each protein was determined for (Hypotonic lysis) high speed pellet fraction, or (Detergent lysis) detergent insoluble fraction.

TABLE II

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<th>Immune complex kinase activity*</th>
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<td>Conditions</td>
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<td>In situ</td>
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* RSV-transformed CECs were separated into fractions which were soluble or insoluble in detergent, or which remained attached to the substratum, and 10-μl aliquots assayed for phosphate transfer to immune serum globulin (44).
‡ Cells lysed directly in radioimmune precipitation assay buffer.
§ Nonimmune rabbit serum was used.

with particulate structures is specific or occurs after cell lysis. The distribution of other major cell proteins did not show such marked dependence on Mg²⁺.

Separation of Cells into Detergent-Soluble and Insoluble Fractions

Since some of the 39-kd protein was particulate regardless of extraction conditions, we tested whether it was in detergent-soluble or insoluble structures. Standard procedures for preparing cytoskeletons involve detergent extraction of cells attached to the substrate, but because transformed cells are poorly adherent it can be difficult to prepare cytoskeletons from them in this way (16). If the cytoskeletons are intended for biochemical rather than microscopic studies, then there is no a priori reason for extracting the cells in situ. For example, Gard et al. (47) scraped cells from the dish and extracted them in suspension to obtain cytoskeletal proteins for two-dimensional gel analysis.

We tested whether trypsinized cells would be suitable for studies of the location of transformation-specific phosphoproteins. 32p-labeled RSV-transformed CECs were prepared for two-dimensional gel electrophoresis either as described (34) directly on the petri dish, or after removal from the dish by

buffer contained Mg²⁺. EDTA is known to solubilize some peripheral membrane proteins (46). Therefore, we compared the proteins of [35S]-methionine labeled CECs and RSV-transformed CECs after homogenization in buffer containing Mg²⁺ (see Materials and Methods). In each case the 46- and 28-kd proteins, and their phosphorylated forms in transformed cells, were soluble, whereas the 39-kd protein, and its phosphorylated form in the transformed cell, was almost completely particulate (Figs. 3 and 4, Table I). Our experiments do not address the question of whether the association of 39-kd protein
Cytoskeletons prepared by extraction of cells attached to the dish, or in suspension, were compared for their content of pp60<sup>Src</sup> by the immune complex kinase assay (Table II). In this particular experiment, the RSV-transformed CECs were sufficiently adherent for their cytoskeletons to remain attached to the petri dish during detergent extraction and washing, and ~95% of the recovered kinase activity was retained in the cytoskeletons (29). When cells were trypsinized or scraped from the dish, <5% of the recovered kinase activity was left on the dish, suggesting that adhesion plaques were efficiently removed by these procedures. The detergent insoluble fraction of cells extracted in suspension contained ~80% of the recovered kinase activity (Table II), suggesting that there was little difference between extraction in suspension or in situ. We also confirmed published results (29, 48) that the distribution of insoluble fraction (see below).

Trypsin digestion. Trypsin treatment lowered the recovery of two 20-kd spots (data not shown) which were previously tentatively identified as pp19<sup>src</sup> variants (r and j, see reference 34). Their loss from trypsinized cells could result from the release of surface-bound virus particles. The 46-, 39-, and 28-kd phosphoproteins were recovered efficiently, suggesting that they are not exposed on the cell surface nor are they in structures which are difficult to remove from the petri dish.

A fractionation procedure was devised in which trypsinized cells were extracted at 4°C for 3 min in detergent solution and the insoluble matter harvested at high speed for 30 sec, under conditions calculated to pellet structures with sedimentation coefficients greater than 30,000S (see Materials and Methods). This procedure gave quantitative recovery of the major protein of chicken fibroblast intermediate filaments, vimentin, in the
pp60src kinase activity between soluble and insoluble fractions was little affected by the addition of 1 mM CaCl₂ or by a combination of 1 mM EGTA and 1 mM GTP, suggesting that the absence or presence of tubulin in the cytoskeletons (21) was not relevant to the distribution of pp60src. For this reason, in subsequent experiments no special precautions were taken to maintain tubulin polymerization.

Next, RSV-transformed CECs were labeled with ³²P, removed from the dish with trypsin, and extracted with detergent in suspension. Detergent soluble and insoluble fractions were prepared and analyzed by two-dimensional gel electrophoresis. Gels were autoradiographed before (not shown) and after incubation in alkali (Fig. 5). The 46-, 39-, and 28-kd phosphoproteins were almost quantitatively extracted into the soluble fraction (Fig. 5A). Some alkali-stable (Fig. 5B) and alkali-sensitive phosphoproteins (not shown) were retained in the insoluble fraction. By comparing the autoradiograph of a gel of mixed detergent soluble and insoluble fractions (Fig. 5C) with that of a gel of cells prepared directly for electrophoresis (not shown) it was confirmed that recovery of the phosphoproteins was not affected by the experimental manipulations.

To study the distribution of the nonphosphorylated forms of the 46-, 39-, and 28-kd phosphoproteins, we fractionated parallel cultures of [³⁵S]methionine-labeled CECs and RSV-transformed CECs by detergent extraction after trypsinization. Two-dimensional gel analysis confirmed that the intermediate filament protein, vimentin, was almost quantitatively retained in the insoluble fraction (Fig. 6A, B, spot 5) as was about one-half the actin (spot 2). Most of the tubulins (spots 4) and vinculin (spot 3) were soluble. The detergent insoluble material from uninfected CECs contained a slightly higher proportion of the cellular vinculin and actin than did the insoluble fraction of transformed CECs (Fig. 7). As expected from the result with ³²P-labeled cells (Fig. 5), the ³⁵S-labeled species corresponding to the 46-, 39-, and 28-kd phosphoproteins were present in the detergent soluble fraction of the transformed cells (Fig. 6A). In addition the precursors of all three phosphoproteins were also soluble, in both normal and transformed CECs (Figs. 6A and 7A).

The low proportion of 39-kd protein which we detected in cytoskeletons was reproducible, but was contrary to the results of other investigators (45, 49; and K. Radke and G. S. Martin, University of California, Berkeley, personal communication). Our extraction procedure differed significantly from theirs with respect to extraction in suspension versus on the dish. Differences in detergent (50) and buffer composition were minor. The presence of Ca²⁺ in Cheng and Chen’s buffer (45) promotes the depolymerization of microtubules, but even without Ca²⁺ we solubilized all the cellular tubulin (Figs. 6 and 7). Therefore we extracted [³⁵S]methionine-labeled CECs on the dish. The nonphosphorylated forms of the 46- and 28-kd phosphoproteins were quantitatively solubilized, but the nonphosphorylated form of the 39-kd protein was now found mostly in the insoluble fraction (Fig. 8). Cytoskeletons prepared in this way appeared otherwise similar to those prepared with cells in suspension, except that there were more weak spots on the two-dimensional gels suggesting less efficient extraction. A larger proportion of vinculin was detergent insoluble, and a high molecular weight protein, probably extracellular fibronectin, was now detected in the detergent insoluble matrix. The distribution of the 39-kd protein was unusual in its dependence on the procedure used to prepare cytoskeletons. We did not test RSV-transformed cells fractionated by lysis on the dish, because of the problem of cell detachment.

The intracellular location of phosphotyrosine-containing proteins, their nonphosphorylated counterparts, and of tyrosine protein kinases was investigated by two types of fractionation procedures, and the results are summarized in Table I. The 46- and 28-kd proteins appear to be soluble cytoplasmic proteins under all conditions. On the other hand, the primary location of the 39-kd protein depended on the conditions. The association of the 39-kd protein with particulate structures showed some dependence on Mg²⁺. Particulate fractions made by homogenization in the presence or absence of Mg²⁺ con-
tained the bulk of the cellular vimentin and about one-half the total actin, suggesting that cytoskeletal fragments and can be recovered by centrifugation. Thus, the Mg\textsuperscript{2+} -dependent association of ~25% of the 39-kd protein with the particulate fraction may reflect divalent cation-dependent association with cytoskeletal or membrane structures. Others have evidence that the ~70% bound in the presence of EDTA may be in the plasma membrane (S. Courtneidge, K. Radke, P. Moss, and G. S. Martin, personal communications.).

After detergent extraction, the location of the 39-kd protein and its phosphoryrosine-containing derivative depended on whether the cells were extracted in suspension or on the substratum. This suggests the 39-kd protein may be weakly associated with a detergent-resistant structure(s) which is either dismantled when cells are removed from the substrate or is too small or too shear sensitive to be recovered by centrifugation (sedimentation coefficient $\leq 30,000S$). Preliminary results of immunofluorescent staining with specific antisera confirm that the 46-kd and 39-kd proteins are not conventional cytoskeletal proteins (E. Nigg and J. A. Cooper, unpublished data.). Anti-46-kd protein serum gives diffuse cytoplasmic staining. Anti-39-kd protein serum gives similar staining of cells permeabilized after fixation, but stains a lattice-like structure in cells permeabilized briefly before fixation. This structure is not coincident with intermediate filaments or microfilaments (Nigg and Cooper, unpublished data.). It is possible that the 38-kd protein may precipitate onto cytoskeletal elements during lysis of cells in situ but not cells in suspension. Such “coating” of filamentous systems has been observed during preparation of cells for microscopic analysis (51). If this association is artifactual, however, it is not merely an example of a basic protein binding to acidic cytoskeletal proteins, since the 28-kd protein, which has the same isoelectric point as the 39-kd protein, does not bind. Further experiments are required to test whether the 39-kd protein binds to specific proteins.

There is thus no obvious pattern to the location of tyrosine protein kinases and their putative substrates. The active forms of all the viral tyrosine protein kinases studied to date are
Figure 7 Fractionation of [35S]methionine-labeled uninfected CECs by detergent extraction after trypsinization from the culture dish. Legend as Fig. 6, except 18.3 x 10^5 cpm (A) and 6.1 x 10^5 cpm (B) were loaded.

cytoskeletal (RSV [29], Abelson virus [52], PRCII virus [53]) but the EGF receptor which has an associated tyrosine protein kinase (54) appears not to be (our unpublished data). The 46- and 28-kd proteins of CECs are not cytoskeletal, nor is an 81-kd protein which is phosphorylated in certain cells in response to EGF (unpublished data). The viral tyrosine protein kinases and the EGF-receptor alike are membrane proteins, but the 46-, 28-, and 81-kd proteins are not. Even though immunofluorescence suggests that vinculin is concentrated in adhesion plaques together with pp60^src (23, 31), much of this protein also appears not to be cytoskeletal (Figs. 7 and 8). This partitioning of tyrosine protein kinases into different subcellular fractions from phosphotyrosine-containing proteins should not be construed to mean that enzyme/substrate relationships do not exist, since diffusible substrates presumably could react with immobilized kinases. Earlier results, which indicated colocalization of pp60^src and vinculin in adhesion plaques (31) and of pp60^src and 39-kd protein in cytoskeletons (29, 45) have been interpreted as circumstantial evidence for enzyme/substrate relationships in vivo. However, it is not clear that the location of pp60^src is essential for transformation, since transformed cells in which pp60^src is not in the plasma membrane have been found (55; L. Rohrschneider, personal communication.). Note also that there is at least one category of substrates which colocalizes with the tyrosine protein kinases; that is the kinases themselves, since all the viral tyrosine protein kinases are themselves phosphorylated on tyrosine (see references in 1).

The location of tyrosine protein kinases in the plasma membrane fraction seems to be quite general. Three viral transforming proteins (RSV pp60^src, Abelson murine leukemia virus P120 [52], and Fujinami sarcoma virus P140 [P. Moss and G. S. Martin, personal communication.]), at least one of their normal cell homologues (pp60^src [10]), and the EGF-receptor associated protein kinase are reportedly membrane proteins. Cell membranes have high levels of tyrosine protein kinase activity in vitro (56). In contrast, phosphotyrosine-containing substrate proteins are commonly not membrane bound. When ^32P-labeled RSV-transformed CECs were homogenized in the presence of Mg^2+ and fractionated by differential centrifugation very little phosphotyrosine was detected in a crude nuclear
fraction, and most of the phosphotyrosine (and phosphoserine and phosphothreonine) was found in the proteins of the high speed supernatant (unpublished observations).

The subcellular locations of the proteins examined here have several consequences. First, attempts to identify substrate proteins colocalized with pp60src in membrane, cytoskeletons or adhesion plaques by incubating these isolated structures with labeled ATP (48, 56, 57) will most likely miss phosphoproteins of the sort described here. Second, although the curious distribution of the 39-kd protein in detergent-insoluble fractions suggests that the latticelike arrangement of the 39-kd protein in cells permeabilized before fixation is due to the association of this protein with membrane fragments, probably derived from the plasma membrane (E. A. Nigg and J. A. Cooper, unpublished results). Since the procedure for permeabilizing cells for immunofluorescence is similar to that used for separating detergent-soluble and insoluble fractions in situ, the localization of the 39-kd protein in detergent-insoluble matrices prepared in situ may at least partly reflect retention of membrane fragments.

Note Added in Proof: Results of continuing immunofluorescence studies suggest that the latticelike arrangement of the 39-kd protein in cells permeabilized before fixation is due to the association of this protein with membrane fragments, probably derived from the plasma membrane (E. A. Nigg and J. A. Cooper, unpublished results). Since the procedure for permeabilizing cells for immunofluorescence is similar to that used for separating detergent-soluble and insoluble fractions in situ, the localization of the 39-kd protein in detergent-insoluble matrices prepared in situ may at least partly reflect retention of membrane fragments.

REFERENCES

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