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Subcellular Location of an Abundant Substrate (p36) for Tyrosine-Specific Protein Kinases

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A 36,000-dalton cellular protein (p36) has been identified previously as an abundant substrate for phosphorylation by tyrosine-specific protein kinases. Since several of the responsible kinases are associated with the plasma membrane, we explored the subcellular distribution of p36. Biochemical fractionations located p36 on the plasma membrane of both normal and retrovirus-transformed cells. Approximately half of the p36 was bound to the membrane with the affinity of a peripheral membrane protein; the remainder was even more tightly bound. The distribution of p36 among subcellular fractions and its affinity for the plasma membrane were not affected by tyrosine phosphorylation. We determined that p36 is synthesized in the soluble compartment of the cell and then moves rapidly to the membranous compartment. Immunofluorescence microscopy with antibodies directed against p36 revealed two distinct distributions of the antigen: (i) a sharply demarcated crenelated pattern within or immediately beneath the plasma membrane, which we presume to be a correlary of the distribution of p36 in biochemical fractionations; and (ii) diffuse staining in a cytoplasmic location that could not be attributed to a specific feature of cytoarchitecture and could not be easily reconciled with the results of biochemical fractionations. Efforts to detect the secretion of p36 were unsuccessful. No evidence was obtained for exposure of p36 on the cell surface, and no changes in localization were observed as a consequence of neoplastic transformation. During the course of this study, we had the opportunity to pursue a previous report that p36 is a component of the enzyme malate dehydrogenase (Rubsamen et al., Proc. Natl. Acad. Sci. U.S.A. 79:228–232, 1982). We were unable to substantiate this claim. We conclude that at least a substantial fraction of p36 is located on the cytoplasmic aspect of the plasma membrane, where it could be well situated to serve as a substrate for several identified tyrosine-specific kinases. But the function of p36 and its role, if any, in neoplastic transformation of cells by retroviruses possessing tyrosine-specific kinases remain enigmatic.

The phenotypic changes which occur when a cell undergoes neoplastic transformation are manifold (17). They range from changes in cell surface properties, such as adhesiveness and contact inhibition, to perturbation of growth control. Transformation can be caused by a variety of agents, including radiation, chemicals, and numerous viruses, but it seems possible that similar or identical biochemical reactions may sustain the neoplastic phenotype in every instance. Recent efforts to elucidate the mechanism(s) of transformation have focused on the oncogenes of retroviruses, whose protein products can both initiate and maintain the transformed phenotype (2).

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The oncogene (v-*src*) of Rous sarcoma virus (RSV) encodes a 60,000-dalton phosphoprotein ($pp60^{v\text{-}src}$) with the enzymatic capacity to phosphorylate tyrosine in protein substrates (7, 8, 15, 16, 19, 23–26). Phosphorylation of tyrosine is a rare modification in normal cells (20), and it may be that an inordinate amount of tyrosine phosphorylation as a consequence of the presence of $pp60^{v\text{-}src}$ is sufficient to transform a cell. The unusual enzymatic activity of $pp60^{v\text{-}src}$ has led to a search for proteins in which phosphotyrosine is newly acquired or increased as a consequence of transformation by RSV.

The first potential substrate (or "target") for $pp60^{v\text{-}src}$ was discovered in chicken cells as a 36,000-dalton protein (p36) which is phosphorylated upon transformation of chicken cells by RSV (32). The kinetics of phosphorylation of

p36 in response to transformation, and the effect of temperature-sensitive mutations in *src* on the phosphorylation, led to the conclusion that the protein may be a direct target of $pp60^{v\text{-}src}$. It was subsequently shown that this phosphorylation could be carried out in vitro by purified $pp60^{v\text{-}src}$ (14) and that phosphorylation both in vivo and in vitro occurred on tyrosine residues (14, 31).

Under certain conditions, p36 is also phosphorylated in normal cells. For example, p36 is one of a number of proteins that are phosphorylated at tyrosine residues during the response of cells to epidermal growth factor (9, 18). The protein kinase responsible for phosphorylation of p36 in uninfected cells has not been identified, but the same tyrosine residue in p36 is phosphorylated during either the response to epidermal growth factor or transformation by v-*src* (9). It therefore appears likely that phosphorylation of p36 may mediate similar events in normal cellular growth and neoplastic transformation. To date, however, efforts to attribute specific components of the transformed phenotype to the phosphorylation of p36 have proven indecisive (29).

In the hope of gaining clues regarding the function of p36 and to further clarify the interaction between p36 and $pp60^{v\text{-}src}$, we sought to identify the location of p36 within normal and transformed cells. Our results with biochemical and immunochemical procedures indicate that some p36 is located on the plasma membrane in the manner of a peripheral membrane protein. It is therefore possible that p36 is in the topographical vicinity of $pp60^{v\text{-}src}$, which is tightly bound to the cytoplasmic aspect of the plasma membrane (12, 21, 39). Our results contrast (but do not conflict) with previous reports that p36 is retained in the detergent-insoluble matrix of the cell (5, 11). We were also unable to confirm a previous report that p36 is a constituent of the enzyme malate dehydrogenase (MDH) (36).

MATERIALS AND METHODS

General procedures. We have described preivously our procedures for propagation and isotopic labeling of cells, immune precipitation, and analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and partial hydrolysis with proteinases (12, 22, 30). Cells used included chicken embryo fibroblasts in secondary or later passages, the Rat 1 line of rat fibroblasts obtained from J. Wyke, the normal rat kidney (NRK) line of rat cells obtained from J. Levy, a line of NRK cells transformed by the Schmidt-Ruppin subgroup D (SR-D) strain of RSV obtained from L. Turek, and mouse NIH 3T3 cells obtained from R. Weinberg.

Subcellular fractionations. Subcellular fractionations were as previously described with minor modifications (12, 22). Cells were removed from dishes by scraping into phosphate-buffered saline (PBS), washed once in PBS, and then hypotonically swollen by incubation for 5 min on ice in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4–5 mM KCl–1 mM $MgCl_2$. After breakage of the cells by Dounce homogenization (15 strokes, tight-fitting pestle), nuclei and unbroken cells were removed by centrifugation at 1,000 × *g* for 5 min. The homogenate was then fractionated into supernatant (cytoplasm) and pellet (membranes) by centrifugation at 100,000 × *g* for 30 min. The membrane pellet was further fractionated on discontinuous sucrose gradients as follows. The membrane was resuspended in 20 mM NaCl–20 mM KCl–20 mM PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)], pH 6.8, by passage through a 26-gauge needle five times and then layered onto a step gradient containing 20, 35, 40, and 50% (wt/vol) sucrose prepared in the same buffer. After centrifugation at 200,000 × *g* for 2.5 h, the membranes were collected from each interface, diluted with at least 10 volumes of the same buffer without sucrose, and pelleted by centrifugation at 100,000 × *g* for 30 min. Material collecting at the 20/35% interface is predominantly derived from the plasma membrane, the 40/50% interface contains mitochondria, lysosomes, and endoplasmic reticulum, and the 35/40% interface is a mixture of plasma membrane and endoplasmic reticulum (12).

Runoff translations. Membrane-bound and cytoplasmic polyribosomes were prepared essentially as described previously (28). Briefly, cells were broken by Dounce homogenization in 5 mM NaCl–1 mM $MgCl_2$–10 mM Tris (pH 8) and centrifuged at 1,000 × *g* for 10 min; the postnuclear supernatant was made to 30% sucrose and fractionated on a discontinuous sucrose gradient of 20, 30, 45, and 60% (wt/vol) sucrose in 1 mM $MgCl_2$–10 mM Tris (pH 7.5) by centrifugation at 100,000 × *g* for 15 h. Membrane-bound polysomes, which were contained on the 45/60% interface, and the pellet of cytoplasmic polyribosomes were washed in 1.5 mM $MgCl_2$–10 mM Tris (pH 7.5), resuspended in water, and incorporated into an in vitro translation system prepared from lysates of rabbit reticulocytes (13).

Purification of p36. p36 was isolated from normal chicken embryo fibroblasts, chicken embryo fibroblasts infected with Schmidt-Ruppin subgroup A RSV, NIH 3T3 cells, and rat fibroblasts transformed by SR-D RSV. The procedure was based on a protocol described by Erikson and Erikson (14). Cells were harvested from monolayer culture by scraping with a rubber policeman. The cells were washed once in PBS and then broken by Dounce homogenization in 25 mM Trishydrochloride (pH 7.5)–1 mM EDTA–1.4 mM 2-mercaptoethanol–0.05% Nonidet P-40. The lysate was centrifuged at 100,000 × *g* for 30 min. The supernatant was applied to a DEAE-cellulose column (DE-52, Whatman) equilibrated with the lysis buffer, and the eluate was passed directly onto an hydroxyapatite column (Bio-Gel HTP; Bio-Rad Laboratories). The coupled columns were washed with lysis buffer until the absorbance of the eluate reached base line; then the DEAE-cellulose column was removed and the hydroxyapatite column was eluted with 200 mM potassium phosphate buffer (pH 7.2)–1 mM EDTA–1.4 mM 2-mercaptoethanol until the absorbance of the eluate again reached base line. p36 was eluted from the column with 400 mM potassium phosphate buffer (pH

7.2)–1 mM EDTA–1.4 mM 2-mercaptoethanol and stored at −70°C. All steps after harvesting of cells were performed at 0 to 4°C. The purity of p36 prepared in this manner and used for immunization and immunofluorescence experiments is evaluated below.

Preparation of antisera to p36. Rabbits were immunized with p36 by subcutaneous injections at multiple dorsal sites. Approximately 100 μg of p36 was used per immunization. The antigen used for primary immunization was purified from normal chicken embryo fibroblasts and given as a 1:1 emulsion with complete Freund adjuvant. We used p36 from a combination of sources in an effort to obtain broadly reactive antisera. Subsequent immunizations of a mixture of p36 from normal and RSV-infected chicken embryo fibroblasts were given every 14 days as 1:1 emulsions with incomplete Freund adjuvant. After the third boost, we obtained antisera that cross-reacted with mammalian (mouse, rat, human) p36. Two additional injections of p36 from SR-D RSV-transformed rat cells were given in an effort to enhance reactivity with mammalian p36. The first was given subcutaneously as described above. The second was given intravenously in PBS 1 month later. This produced the high-titer sera used for immunofluorescence studies.

Immunofluorescence microscopy. Cells grown on glass cover slips were washed with PBS and fixed with fresh 3% paraformaldehyde in PBS for 20 min at room temperature. After brief rinses in PBS, the cells were permeabilized by 0.2% Triton X-100 at room temperature for 5 min. Antisera were incubated on the cells at dilutions of 1:20 to 1:70 for 30 min at room temperature. After extensive washes in PBS, the cover slips were stained with rhodamine-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories). Cover slips were mounted wet in 50% glycerol in PBS. For staining of extracellular antigen only, the cells were incubated with antibodies and washed on ice, fixed with paraformaldehyde, and stained with the rhodamine conjugate.

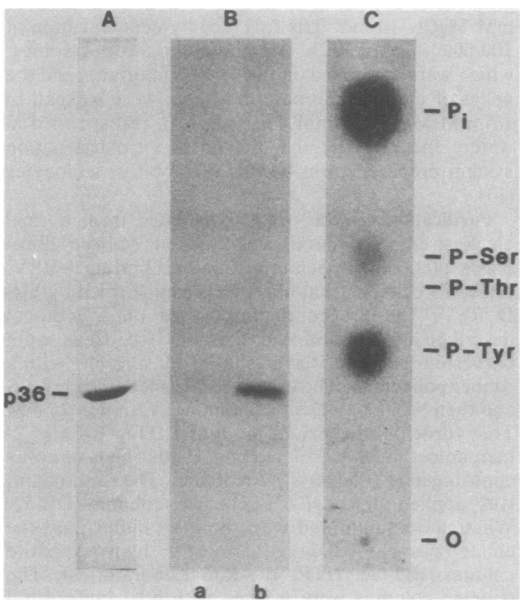

FIG. 1. Validation of antisera to p36. (A) A 10-μg amount of purified p36 was analyzed by sodium dodecylsulfate-polyacrylamide gel electroploresis with Coomassie blue staining. (B) ^{32}P-labeled p36 was immunoprecipitated from NRK (lane a) and SR-D-transformed (lane b) rat cells. (C) Phosphoamino acid analysis of ^{32}P-labeled p36 shown in (B), lane b.

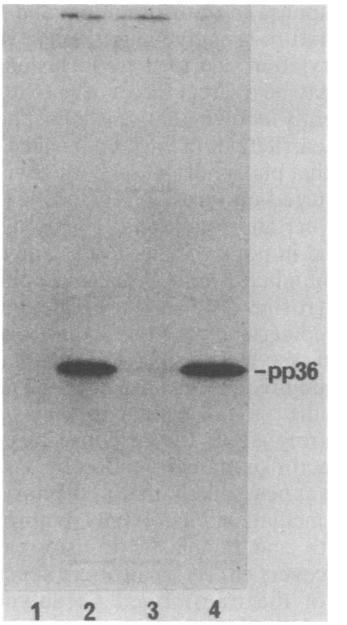

FIG. 2. Distribution of ^{35}S-labeled p36 among subcellular fractions. Rat 1 cells were labeled with [^{35}S]methionine for 3 h, fractionated, and then subjected to immune precipitation. The sample in lane 1 was precipitated with normal rabbit serum; the remaining samples were precipitated with rabbit anti-p36. Lane 1, Unfractionated homogenate; lane 2, unfractionated homogenate; lane 3, S100; lane 4, P100.

Determination of MDH activity. MDH activity was assayed essentially according to the procedure of Siegel and Bing (37) and expressed in international units. Fractions were diluted with 0.1 M potassium phosphate buffer, pH 7.5, containing 0.066 mg of NADH per ml to a final volume of 0.29 ml and allowed to stand for 30 min on ice. A 0.010-ml portion of 1.0-mg/ml oxaloacetic acid was then added. The oxidation of NADH was followed spectrophotometrically at 340 nm.

RESULTS

Validation of antisera to p36. p36 was purified as described under Materials and Methods. A typical p36 preparation used for immunization is shown in Fig. 1A. Antisera from rabbits immunized with the purified protein specifically precipitated a protein of 36,000 daltons (see below; Fig. 2). The authenticity of this protein was confirmed as follows: NRK and RSV-trans-

formed rat cells were labeled with $^{32}P_i$, and p36 was immunoprecipitated from the cell lysates. As expected, p36 was not phosphorylated in the NRK cells (Fig. 1B, lane a), but was strongly phosphorylated in the RSV-transformed cells (lane b). Phosphoamino acid analysis of the ^{32}P-labeled protein showed that phosphotyrosine was the major phosphoamino acid present (Fig. 1C). Some phosphoserine was also observed. In addition, mapping with V8 protease produced patterns similar to published maps of p36 (31). We conclude that our antiserum recognizes authentic p36 and its phosphorylated form(s).

p36 is a membrane protein. We used biochemical fractionation of cellular organelles to explore the localization of p36 in uninfected rat cells labeled with [^{35}S]methionine. Figure 2 shows an example of such a fractionation. Immune precipitation of the cell homogenate recovered p36 as anticipated (Fig. 2, lane 2). The majority of the protein fractionated into the crude membrane pellet (lane 4), with <10% being found in the cytoplasm (lane 3). The same distribution of the [^{35}S]methionine-labeled protein was also seen in rat cells transformed by SR-D RSV (Fig. 3A). The majority of the protein was in the membrane fraction (Fig. 3A, lane 5), with very little if any in the cytoplasm (lane 4). Thus, transformation did not grossly affect the subcellular distribution of the protein. Appreciable quantities of p36 (ca. 38% of the total recovered) were found in the crude nuclear pellet (lane 3). We attribute this to the presence of unbroken cells and sheets of membrane, but an alternate explanation is possible (see Discussion). Whatever the explanation, virtually all of the p36 could be removed from the nuclear fractions by washes with 5 mM KCl–1 mM $MgCl_2$–0.5% Nonidet P-40–0.1% deoxycholate–20 mM HEPES buffer, pH 7.4. We conclude that p36 is not likely to be a nuclear protein. Analysis with immunofluorescence sustained this conclusion (see below).

Approximately 10% of p36 is phosphorylated in cells transformed by RSV (14, 32). We examined the distribution of phosphorylated p36 (pp36) by subcellular fractionation of RSV-transformed rat cells labeled with ^{32}P (Fig. 3B). Most of pp36 was membrane associated (lane 5), with very little being in the soluble fraction (lane 4). The faint band migrating immediately behind pp36 is an electrophoretic variant of the protein (R.R., unpublished data).

The membrane pellet prepared in this way contains mitochondria, lysosomes, Golgi apparatus, endoplasmic reticulum, and plasma membrane. This heterogeneous population was fractionated on discontinuous gradients of sucrose and then immune precipitated to determine more

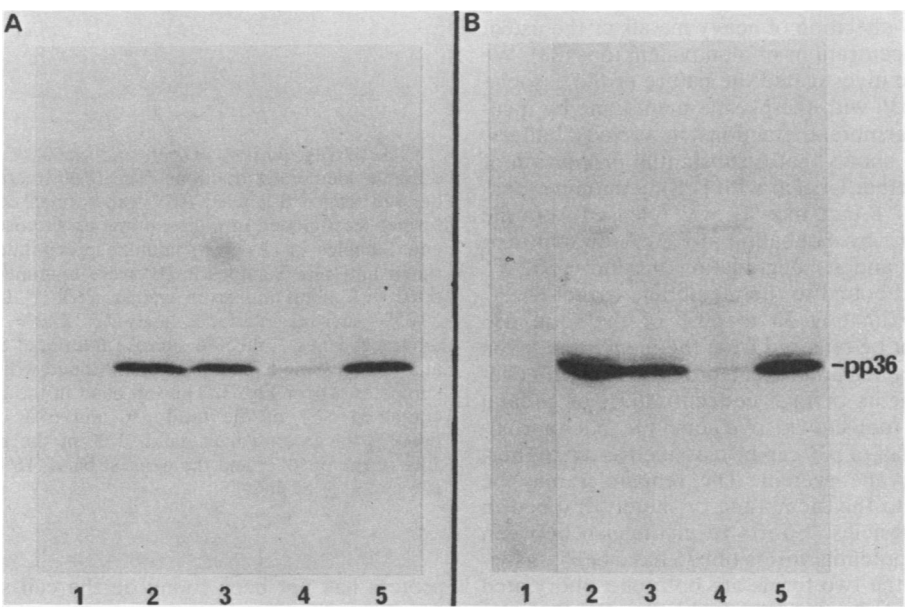

FIG. 3. Distribution of ^{35}S- and ^{32}P-labeled p36 among subcellular fractions. NRK cells transformed by SR-D RSV were labeled with [^{35}S]methionine (A) or ^{32}P (B), fractionated, and then subjected to immune precipitation. The samples in lanes 1 were immune precipitated with normal rabbit serum; the rest were precipitated with rabbit anti-p36. Lanes 1, Unfractionated homogenate; lanes 2, unfractionated homogenate; lanes 3, nuclear fraction; lanes 4, S100; lanes 5, P100. Suitable autoradiographic exposures were scanned with a densitometer to obtain the numerical results given in the text.

precisely the subcellular localization of ^{35}S-labeled p36. A representative analysis of fractions from RSV-transformed cells is shown in Fig. 4A. The majority of p36 (55 to 60%) was found in the fraction enriched for plasma membrane (lane 1); the remainder was in the intermediate band (lane 2) containing a mixture of plasma membrane and smooth endoplasmic reticulum (27%) and in the densest band (lane 3) containing rough endoplasmic reticulum (16%). The precise composition of each of the fractions has been reported previously (12). In the present experiments, the validity of the fractionations was assessed by monitoring the presence of pp60$^{v\text{-}src}$ (Fig. 4B) because its distribution among the fractions is firmly established (12). We found that p36 and pp60$^{v\text{-}src}$ fractionated almost identically (see legend to Fig. 4) and therefore concluded that p36 is probably attached to the plasma membrane. The subcellular distribution of p36 was similar in uninfected and RSV-transformed cells and in cells labeled with either [^{35}S]methionine or ^{32}P (Fig. 4; our unpublished data).

p36 appears to be a peripheral membrane protein. The association between proteins and membranes generally takes either of two forms: integral proteins are inserted into the lipid bilayer and can be released only by disruption of the membrane with detergents, whereas peripheral proteins are attached to the surface of the membrane, or to its attached cytoskeletal elements, and can be dissociated from the membrane by chelation of heavy metals or the use of high concentrations of monovalent ions (38). We therefore investigated the nature of the association of p36 with the plasma membrane by incubating membrane fractions in various buffers. Figure 5 shows that a substantial proportion of p36, whether labeled with [^{35}S]methionine (panel A) or ^{32}P (not shown), was released from the membrane by incubation in EDTA. In contrast, pp60$^{v\text{-}src}$ and its degradation product pp52$^{v\text{-}src}$ remained bound to the membrane (panel B).

Approximately 30 to 60% of the total p36 could not be released from the membrane, even by protracted and repeated treatments with chelating agents or high concentrations of sodium chloride (not shown). We conclude that approximately half of p36 can be classified as a peripheral membrane protein. The remainder may be integral to the membrane or merely trapped in sealed vesicles. Efforts to distinguish between the two potential forms of p36 have been unsuccessful; the two forms are both phosphorylated and cannot be distinguished by partial hydrolysis with proteases (data not shown).

Growth medium harvested from either normal or RSV-transformed cells contained no detectable p36. We therefore conclude that p36 is not secreted in appreciable quantities. Similarly, the protein has not been found on the cell surface (see below).

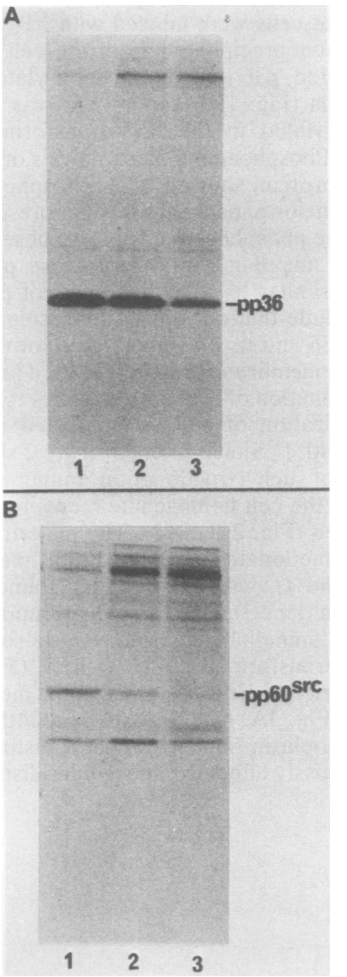

FIG. 4. Distribution of p36 and pp60$^{v\text{-}src}$ among different membrane fractions. The P100 fraction was derived from ^{35}S-labeled RSV-transformed cells and further fractionated in a discontinuous sucrose gradient. Samples in (A) were immune precipitated with rabbit anti-p36. Samples in (B) were immunoprecipitated with rabbit antiserum against pp60$^{v\text{-}src}$. Lanes 1, 20/35% sucrose interface; lanes 2, 35/40% sucrose interface; lanes 3, 40/50% sucrose interface. Suitable autoradiographic exposures were scanned with a densitometer, as for Fig. 3. The top band in the gradient contained 57% of the total p36 and 65% of total pp60^{src}; the intermediate band, 27% of the p36 and 20% of the pp60^{src}; and the densest band, 16% of the p36 and 15% of pp60^{src}.

p36 is synthesized in the cytoplasm. We first attempted to locate the synthesis of p36 within the cell by the use of pulse-chase labeling. However, even with labeling times as short as 2 min, most of the labeled p36 was found associat-

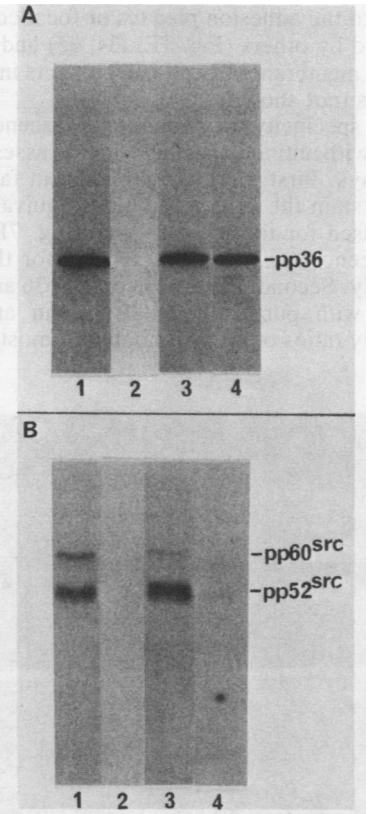

FIG. 5. Extraction of p36 and pp60$^{v\text{-}src}$ from membrane vesicles. RSV-transformed NRK cells were labeled with [^{35}S]methionine, and crude membranes (P100) were prepared and resuspended in the buffers indicated below. After incubation for 1 h at 4°C, the membranes were recentrifuged and both pellets and supernatants were analyzed for the presence of p36 (A) and pp60$^{v\text{-}src}$ (B). Lanes 1, Membrane pellet after incubation in 5 mM KCL–20 mM PIPES, pH 6.8; lanes 2, material released from membrane after incubation in 5 mM KCl–20 mM PIPES, pH 6.8; lanes 3, membrane pellet after incubation in 5 mM KCl–10 mM EDTA–20 mM PIPES, pH 6.8; lanes 4, material released from membrane after incubation in 5 mM KCl–10 mM EDTA–20 mM PIPES, pH 6.8.

ed with plasma membranes (10% of pp36 was soluble after such a pulse, compared with <2% at steady state). There have been no previous reports of peripheral membrane proteins being synthesized on membrane-bound polysomes. We therefore sought an alternative means of assessing the site of p36 synthesis. To do this, postnuclear supernatants from RSV-transformed rat cells were fractionated on sucrose gradients such that cytoplasmic and membrane-bound polyribosomes were separately obtained. The fractions were then supplemented with a reticulocyte lysate for translation, protein synthesis was allowed to continue in vitro, and the products were examined for p36 by immunoprecipitation (Fig. 6). Polyribosomes which derived from the cytoplasm (lane 4), but not those from the membrane fraction (lane 2), contained mRNA for p36. Thus, p36 appears to be made in the cytoplasm and then transported extremely rapidly to the membrane.

Localization of p36 by immunofluorescent microscopy. We sought further information about the subcellular localization of p36 by the use of immunofluorescent microscopy. To effectively visualize structural details, we used cells 2 to 6 h after plating, when spreading and extension of the cells were maximal. Similar results were obtained, however, when cells were used 24 h after trypsinization.

Viable cells stained with antibody without prior permeabilization gave no specific immunofluorescence, suggesting that p36 is not exposed on the surface of cells. When stained after fixation and permeabilization, uninfected rat cells (NRK) displayed two forms of immunofluorescence (Fig. 7A): (i) a diffuse pattern most apparent around the nucleus and fading toward the periphery of the cell (dark vacuoles were often superimposed on this pattern, and the nucleus was similarly not stained); and (ii) a sharp crenelated pattern subjacent to the edges of the cells, usually in regions found to be lamellar projections, and ruffles of the plasma membrane by phase microscopy.

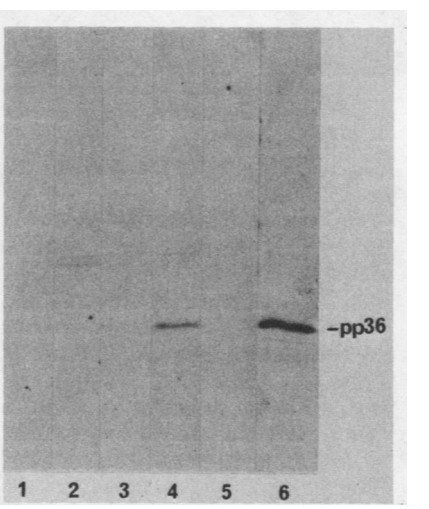

FIG. 6. Site of biogenesis of p36. Membrane-bound and cytoplasmic polyribosomes were prepared from RSV-transformed NRK cells by differential centrifugation and incorporated into an in vitro translation system. Lanes 1, 3, and 5 were analyzed by immune precipitation with normal rabbit serum. Lanes 2, 4, and 6 were analyzed by immune precipitation with rabbit anti-p36 serum. Lanes 1 and 2, Membrane-bound polyribosomes; lanes 3 and 4, cytoplasmic polyribosomes; lanes 5 and 6, total cellular RNA.

Cells transformed by RSV were more difficult to examine because of their rounded morphology and their failure to spread well on the substratum. As a consequence, the transformed cells frequently displayed only a diffuse and poorly defined cytoplasmic fluorescence. In occasional examples, however, well-spread cells showed the crenelated peripheral staining in flower-like protrusions and ruffles of the plasma membrane (Fig. 7D). The localization of p36 in the transformed cells was distinctly different from that of $pp60^{v\text{-}src}$, which was found in focal areas on the undersurface of cells that presumably correspond to the adhesion plaques or focal contacts reported by others (Fig. 7E) (34, 35) and in the plasma membranes at cell-cell contacts in dense cultures (not shown).

The specificity of immunofluorescence obtained with antibody against p36 was assessed in two ways. First, normal rabbit serum failed to visibly stain the cells at dilutions equivalent to those used for the p36 antibody (Fig. 7B); the fluorescence was therefore specific for the p36 antibody. Second, incubation of the p36 antisera (5 μl) with purified p36 (20 μg) in antigen/antibody ratios of ca. 40:1 abolished most of the

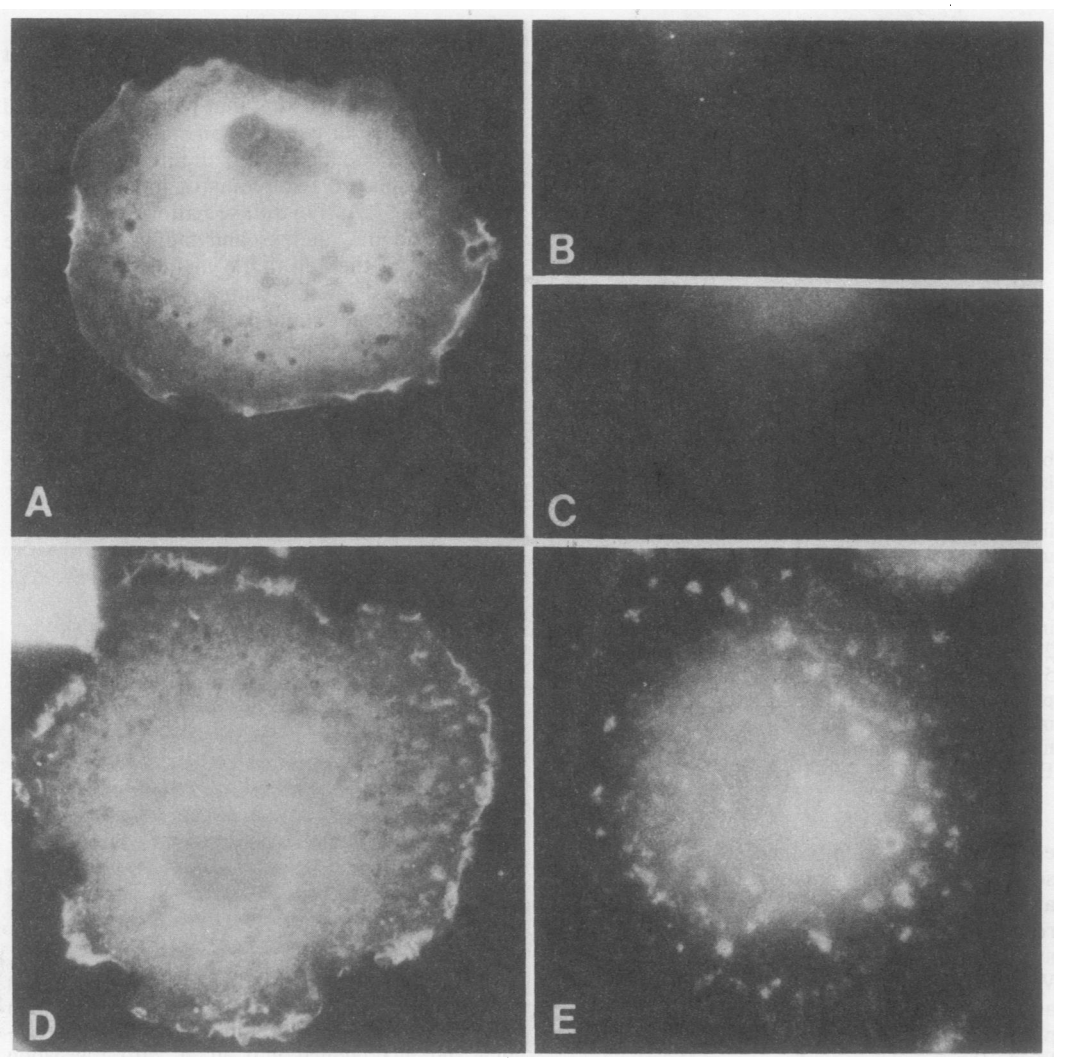

FIG. 7. Indirect immunofluorescence for p36 in normal and RSV-transformed cells. NRK cells (A, B, and C) or RSV-transformed NRK cells (D and E) were fixed and permeabilized as detailed in the text and stained with anti-p36 antiserum (A, D), normal rabbit serum (C), antigen-blocked anti-p36 serum (B), and anti-$pp60^{v\text{-}src}$ serum obtained from tumor-bearing rabbits and made specific by adsorption with detergent-disrupted virions (E). The fluorescent speckles seen in (B) may represent immune complexes not removed by centrifugation at 3,000 × *g* after the blocking reaction (6-h incubation with mixing at room temperature).

staining of normal and transformed cells (see Fig. 7C); the staining was therefore probably specific for the p36 immunogen used to prepare the antisera.

We conclude that the membrane location attributed to p36 by the results of biochemical fractionations is also apparent in the peripheral staining of cells by specific immunofluorescence. The biochemical equivalent of the diffuse cytoplasmic staining is less apparent (see Discussion).

Search for MDH in preparations of p36. Rubsamen et al. (36) have reported that p36 is a cytosolic form of MDH. When fractions from our original rpeparations of p36 were tested for MDH activity, however, >95% of the activity present in the crude lysate and S-100 fractions was recovered in the 200 mM potassium phosphate eluate (HAP 200) of the hydroxyapatite column. Little or no MDH activity was found in the 400 mM eluate (HAP 400) that contained virutally all of the p36. To rule out any loss of activity due to instability of the protein, a small-scale preparation of p36 was made and the fractions were assayed immediately (Fig. 8A). The indicated quantities of MDH activity from various fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. There was no correlation between MDH activity and the 36,000-dalton band present in the HAP 400 fraction. Purer preparations of p36 from a variety of cells showed no detectable MDH activity (Fig. 8B).

DISCUSSION

Characterization of purified p36. An abundant 36,000-dalton protein (p36) found in avian and mammalian cells appears to be a substrate for phosphorylation on tyrosine by both retroviral and cellular protein kinases (9, 10, 14, 18, 31). At least several of the responsible kinases are membrane proteins (2, 6, 12, 21, 34, 39, 40). The subcellular locations of p36 and its phosphorylated form were therefore of interest.

To facilitate the localization of p36, we first purified the protein and prepared antibodies against it as described previously (14). We documented the identity of the purified protein with the following evidence: (i) peptide maps obtained by partial proteolysis of the purified protein matched those reported for p36 (14, 31); (ii) a fraction of the protein purified from RSV-transformed cells (but not from uninfected cells) was phosphorylated on tyrosine (data not shown); and (iii) the antisera prepared by using the purified protein as an immunogen precipitated a 36,000-dalton protein that produced the peptide map of authentic p36 and that was phosphorylated on tyrosine in cells transformed by RSV.

A recent report that p36 is a component of a cytosolic form of MDH (36) prompted us to test our preparations of p36 for this enzymatic activity. Our most extensively purified material displayed no detectable MDH activity. In addition, when fresh material was prepared and MDH activity was followed throughout, the bulk of the enzymatic activity separated from p36 early in the fractionation scheme. We estimate that if p36 is indeed a form of MDH, it must represent <0.5% of the total MDH activity in our crude extracts. The abundance of p36 and the very low levels of MDH detectable in even our incompletely purified preparations could only be explained by extremely feeble enzymatic activity (i.e., low "turnover" values) or preferential inactivation of any MDH attributable to p36.

Subcellular localization of p36 by biochemical fractionation. We used rate-zonal and isopycnic centrifugation to fractionate subcellular organelles. Since the procedures were identical to those used previously to localize $pp60^{v\text{-}src}$ (12), we exploited the presence of $pp60^{v\text{-}src}$ in extracts from RSV-infected cells as a convenient internal control for the efficacy of the fractionations. We found that p36 and $pp60^{v\text{-}src}$ were enriched to equivalent extents in fractions containing primarily plasma membranes. Similar results were obtained for p36 with either uninfected or RSV-transformed cells and for either p36 or its phosphorylated form in transformed cells. We therefore conclude that p36 is associated with the plasma membrane and that the extent of the association is not altered appreciably by transformation of the cell or by phosphorylation of p36.

When we examined the chemical nature of the association between p36 and the plasma membrane, however, a perplexing duality emerged. Approximately half of p36 displayed properties of a peripheral membrane protein; the remainder behaved as if it were integral to the membrane. In the absence of more detailed studies, we cannot discern the significance of these findings. It is possible that the ostensibly integral form of p36 was merely trapped within sealed membrane vesicles. Alternatively, the duality of affinities between p36 and the membrane might reflect the possibility that p36 forms dimers (14; R.R., unpublished data): one half of the dimer might anchor the other half to the membrane and thus be more tightly bound to the membrane. Whatever the explanation, we could not distinguish between the tightly and loosely bound forms of p36 by size, peptide maps, or extent of phosphorylation.

We found no evidence for secretion of p36 from the cell or exposure of the protein on the

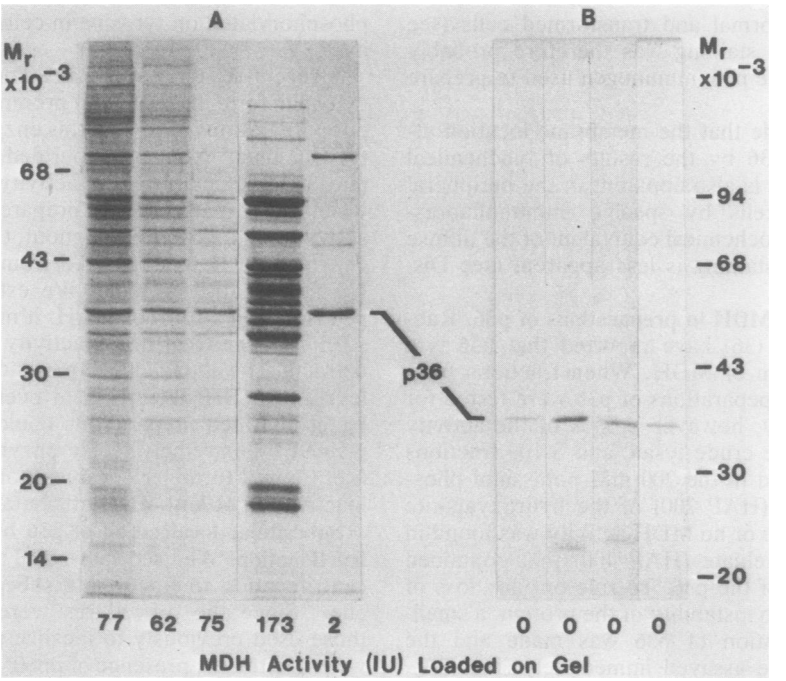

FIG. 8. Analysis of MDH activity in p36 fractions. (A) MDH activity in p36 fractions from rapid purification. Numbers at the bottom of lanes indicate MDH activity present in the sample loaded on the gel. The fractions (left to right) are crude lysate, S-100, DEAE-cellulose flow-through, 200 mM potassium phosphate buffer eluate of the hydroxyapatite column, and 400 mM potassium phosphate buffer eluate. (B) Purified p36 from (left to right) normal chicken embryo fibroblasts, SR-D-transformed chick fibroblasts, NRK cells, and SR-D-transformed rat cells.

external surface of the cell. Nevertheless, treatment of membrane vesicles with proteases led to partial hydrolysis of p36 (S.C., unpublished data), much as for pp60$^{v\text{-}src}$ (22). These findings led to our provisional conclusion that p36, like pp60$^{v\text{-}src}$, is attached to the cytoplasmic aspect of the plasma membrane (22). This potential congruence of locations was not reflected in a more specialized situation of pp60$^{v\text{-}src}$: we found no concentration of p36 in adhesion plaques/focal contacts that were clearly delineated by immunofluorescence microscopy with antisera against pp60$^{v\text{-}src}$. We do not regard these observations as contradictory, since it is unlikely that more than a small proportion of pp60$^{v\text{-}src}$ (ca. 10%) is actually congregated in adhesion plaques (35).

Localization of p36 by immunofluorescence microscopy. It is doubtful that we achieved complete purification of p36. The antisera we prepared against p36 are therefore unlikely to be monospecific. The fluorescent staining obtained with the antisera can nevertheless be safely attributed to a reaction with p36. We obtained extensive reduction in the immunofluorescence by blocking the antibody with a 40-fold excess of antigen; no protein other than p36 was present in the preparation of blocking antigen in sufficient quantity to occupy the bulk of the specific antibodies (Fig. 7; R.R., unpublished data).

Immunofluorescence revealed two unfamiliar staining patterns for p36. (i) Strong fluorescence was distributed diffusely over the cytoplasm in the vicinity of the nucleus, fading toward the periphery of the cell. No structural details could be perceived, such as would be expected if the staining were due to antigen in cytoskeletal structures, Golgi apparatus, or endoplasmic reticulum. We doubt that this form of staining detected an antigen in the plasma membrane because the plane of focus used to perceive the staining was not suitable for surface fluorescence; in addition, the staining failed to obscure cytoplasmic vacuoles and nuclei, which were unstained. (ii) A sharply demarcated, crenelated pattern of staining appeared within or immediately beneath the plasma membrane, particularly in ruffles and other regions of membrane extension. We presume that this staining reflects, at least in part, the membranous p36 identified by biochemical fractionation. The pattern of staining is unfamiliar to us, however, and we have been unable to attribute it to a previously recognized detail of cytoarchitecture.

The results obtained with biochemical frac-

tionation and immunofluorescent microscopy are difficult to reconcile. The preponderance of immunofluorescence appeared as cytoplasmic staining, most intense in the vicinity of the nucleus. By contrast, much of immunoprecipitable p36 was contained in fractions enriched for plasma membrane, and very little of the protein appeared in even the crudest of cytosol preparations. It is probably inappropriate to make quantitative comparisons between these disparate results. First, we are unable to assign the diffuse staining to a specific subcellular location. Second, the factors that determine the signal strength of immunofluorescence from different locales in the cell are obscure. Third, many membrane proteins fail to give decisive patterns of immunofluorescence even if their localization is otherwise well established. The pattern of fluorescence obtained with $pp60^{v\text{-}src}$ provides an apt example: the protein has been localized almost entirely to the plasma membrane by both biochemical fractionation (12, 21) and immunoelectron microscopy (39), whereas immunofluroescence has given only occasional suggestions of diffuse membrane staining and has instead detected $pp60^{v\text{-}src}$ in both perinuclear aggregates (33) and adhesion plaques (34).

Crude nuclear fractions contained appreciable quantities of p36. We have not been able to explore the nature of this affiliation. It could be due merely to contamination of the nuclear fraction with unbroken cells and sheets of membrane, or it could represent the p36 responsible for the perinuclear staining in immunofluorescence.

Biogenesis of p36. We were unable to dissociate the synthesis of p36 from membranous structures by the use of pulse-chase labeling. The majority of the protein was found in membrane fractions after labeling periods as brief as 2 min. We therefore turned to the procedure of "runoff translation," in which polyribosomes are first isolated and the synthesis of nascent proteins is then completed in vitro (28). By this means, we were able to show that p36 is synthesized on soluble rather than membrane-bound polyribosomes. The results obtained by pulse-labeling of intact cells therefore suggest that the completed protein moves quickly and with great specificity to the plasma membrane. It remains possible, however, that a portion of the membrane binding of p36 detected after pulse-labeling reflects adventitious interactions occurring during subcellular fractionation. If so, the interactions must be quite strong because over half of the pulse-labeled p36 could not be dissociated from membranes by high concentrations of monovalent cation or by chelating agents (S.C., unpublished data).

Is p36 in the cytoskeleton? Previous work has shown that p36 remains in the insoluble "cytoskeleton" prepared by treatment of cultured cells with nonionic detergent (5, 11). We doubt that this finding is in conflict with our present results. The composition of insoluble cytoskeleton is often ambiguous: integral membrane proteins have been recovered in high yield from such preparations (1). In addition, proteins may interact with more than one domain of cytoarchitecture. For example, the protein encoded by the oncogene of Abelson murine leukemia virus spans the plasma membrane (40), yet remains attached to the cytoskeleton after treatment with nonionic detergent (3). Recent studies have revealed the existence of a proteinaceous framework or "endoskeleton" on the cytoplasmic aspect of the plasma membrane of erythrocytes (4) and less specialized cells (27). Perhaps p36 is part of, or is anchored to, this endoskeleton.

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LITERATURE CITED

1. **Ben Ze'ev, A., A. Duerr, F. Solomon, and S. Penman.** 1979. The outer boundary of the cytoskeleton: a lamina derived from the plasma membrane proteins. Cell 17:859–865.
2. **Bishop, J. M., and H. E. Varmus.** 1982. Functions and origins of retroviral transforming genes, p. 999–1108. *In* R. A. Weiss, N. Teich, H. E. Varmus, and J. Coffin (ed.), Molecular biology of tumor viruses, part III. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Boss, M. A., G. Dreyfuss, and D. Baltimore.** 1981. Localization of the Abelson murine leukemia virus protein in a detergent-insoluble subcellular matrix: architecture of the protein. J. Virol. 40:472–481.
4. **Branton, D., C. M. Cohen, and J. Tyler.** 1981. Interaction of cytoskeletal proteins on the human erythrocyte membrane. Cell 24:24–32.
5. **Cheng, Y.-S. E., and L. B. Chen.** 1981. Detection of phosphotyrosine-containing 36,000 dalton protein in the framework of cells transformed with Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 78:2388–2392.
6. **Cohen, S., G. Carpenter, and L. King.** 1980. Epidemal growth factor-receptor-protein kinase interactions. J. Biol. Chem. 255:4834–4842.
7. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. 75:2021–2024.
8. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus-transforming protein, $pp60^{src}$, shows protein kinase activity specific for tyrosine. Nature (London) 285:167–169.
9. **Cooper, J. A., and T. Hunter.** 1981. Similarities and differences between the effects of epidermal growth factor and Rous sarcoma virus. J. Cell. Biol. 91:878–883.
10. **Cooper, J. A., and T. Hunter.** 1981. Four different classes of retroviruses induce phosphorylation of tyrosine present in similar cellular proteins. Mol. Cell. Biol. 1:394–407.
11. **Cooper, J. A., and T. Hunter.** 1982. Discrete primary

locations of a tyrosine protein kinase and of three proteins that contain phosphotyrosine in virally transformed chick fibroblasts. J. Cell. Biol. 94:287–296.
12. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$), are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. 77:3783–3787.
13. **Dahl, H.-H. M., and C. Dickson.** 1979. Cell-free synthesis of mouse mammary tumor virus Pr77 from virion and intracellular mRMA. J. Virol. 29:1131–1141.
14. **Erikson, E., and R. L. Erikson.** 1980. Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus transforming gene product. Cell 21:829–836.
15. **Erikson, R. L., M. S. Collett, E. Erikson, and A. F. Purchio.** 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic-AMP-independent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 76:6260–6264.
16. **Gilmer, T. M., and R. L. Erikson.** 1981. The Rous sarcoma virus transforming protein $pp60^{src}$ expressed in *E. coli* functions as a protein kinase. Nature (London) 294:771–773.
17. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology. Plenum Publishing Corp., New York.
18. **Hunter, T., and J. A. Cooper.** 1981. Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Cell 24:741–752.
19. **Hunter, T., and B. M. Sefton.** 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 77:1311–1315.
20. **Hunter, T., B. M. Sefton, and J. A. Cooper.** 1981. Phosphorylation of tyrosine: its importance in viral transformation and normal cell metabolism, p. 1189–1202. *In* O. M. Rosen and E. G. Krebs (ed.), Cold Spring Harbor conferences on cell proliferation. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Krueger, J. G., E. Wang, and A. R. Goldberg.** 1979. Evidence that the *src* gene product of Rous sarcoma virus is membrane associated. Virology 101:25–40.
22. **Levinson, A. D., S. A. Courtneidge, and J. M. Bishop.** 1981. Structural and functional domains of the Rous sarcoma virus transforming protein ($pp60^{src}$). Proc. Natl. Acad. Sci. U.S.A. 78:1624–1628.
23. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell 15:561–572.
24. **Levinson, A. D., H. Oppermann, H. E. Varmus, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. 255:11973–11980.
25. **Maness, P. F., E. Hansjorg, M. E. Greenberg, M. O'Farrell, W. E. Gall, and G. M. Edelman.** 1979. Characteristics of the protein kinase activity of avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. 76:5028–5032.
26. **McGrath, J. P., and A. D. Levinson.** 1982. Bacterial expression of an enzymatically active protein encoded by the RSV *src* gene. Nature (London) 295:423–426.
27. **Mescher, M. F., M. J. L. Jose, and S. P. Balk.** 1981. Actin-containing matrix associated with the plasma membrane of murine tumors and lymphoid cells. Nature (London) 289:139–144.
28. **Morrison, T. G., and H. F. Lodish.** 1975. Site of synthesis of membrane and non-membrane proteins of vesicular stomatitis virus. J. Biol. Chem. 250:6955–6962.
29. **Nakamura, K. D., and M. J. Weber.** 1982. Phosphorylation of a 36,000 M_r cellular protein in cells infected with partial transformation mutants of Rous sarcoma virus. Mol. and Cell. Biol. 2:147–153.
30. **Oppermann, H., A. D. Levinson, L. Levintow, H. E. Varmus, J. M. Bishop, and S. Kawai.** 1981. Two cellular proteins that immunoprecipitate with the transforming protein of the Rous sarcoma virus. Virology 113:736–751.
31. **Radke, K., T. Gilmore, and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell 21:821–828.
32. **Radke, K., and G. S. Martin.** 1979. Transformation by the Rous sarcoma virus: effects of *src* gene expression on the synthesis and phosphorylation of cellular polypeptides. Proc. Natl. Acad. Sci. U.S.A. 78:5212–5216.
33. **Rohrschneider, L. R.** 1979. Immunofluorescence on avian sarcoma virus-transformed cells: localization of the *src* gene product. Cell 16:11–24.
34. **Rohrschneider, L. R.** 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product. Proc. Natl. Acad. Sci. U.S.A. 77:3514–3518.
35. **Rohrschneider, L., M. Rosok, and K. Shriver.** 1982. Mechanism of transformation by Rous sarcoma virus: events within adhesion plaques. Cold Spring Harbor Symp. Quant. Biol. 46:953–965.
36. **Rubsamen, H., K. Saltenberger, R. R. Friis, and E. Eigenbradt.** 1982. Cytosolic malic dehydrogenase activity is associated with a putative substrate for the transforming gene product of the Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 79:228–232.
37. **Siegel, A., and R. J. Bing.** 1956. Plasma enzyme activity in myocardial infarction in dog and man. Proc. Soc. Exp. Biol. Med. 91:604–610.
38. **Singer, S. J.** 1974. The molecular organisation of membranes. Annu. Rev. Biochem. 43:805–833.
39. **Willingham, M. C., G. Jay, and I. Pastan.** 1979. Localisation of the ASV *src* gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell 18:125–134.
40. **Witte, O., N. Rosenberg, and D. Baltimore.** 1979. Preparation of syngeneic regressor serum reactive with the unique determinants of the Abelson murine-leukemia virus-encoded p120 protein at the cell surface. J. Virol. 31:776–784.