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Biochemistry

Association of pp36, a phosphorylated form of the presumed target protein for the src protein of Rous sarcoma virus, with the membrane of chicken cells transformed by Rous sarcoma virus

(phosphoprotein/membrane association/tumor cells)

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ABSTRACT **A cellular protein with a molecular mass of approximately 36 kilodaltons is the presumed target protein of the src protein [the transforming protein encoded by Rous sarcoma virus (RSV)]. The cellular location of the phosphorylated 36-kilodalton protein (pp36) in chicken embryo fibroblasts transformed by the Schmidt–Ruppin strain of RSV has been investigated. In these studies, two-dimensional electrophoresis was used for detection of the phosphoproteins in total cell extracts and also in fractionated subcellular components. We conclude that pp36 is localized in the plasma membrane, on the basis of the following observations. (*i*) Fractionation of ^{32}P-labeled cell extracts showed that pp36 is almost exclusively localized in the crude membrane fraction and no appreciable amount was found in nuclear or cytoplasmic fractions. (*ii*) On further fractionation of the crude membrane fraction, pp36 was localized mostly in the plasma membrane rather than in other membranous fractions. (*iii*) Isolated plasma membrane by itself phosphorylated the 36-kilodalton protein on incubation with [γ-^{32}P]ATP.**

A recent investigation has shown that the *src* gene of Rous sarcoma virus (RSV) encodes a 60-kilodalton (kDal) protein, pp60$^{v\text{-}src}$ (1). This protein was identified by immunoprecipitation from RSV-transformed cells with sera obtained from rabbits bearing tumors induced by RSV (2, 3). In addition, pp60$^{v\text{-}src}$ was synthesized in an *in vitro* system programmed by RSV virion RNA (4, 5). It has been established that pp60$^{v\text{-}src}$ is a protein kinase (2, 3, 6–8) and that it has the specificity of phosphorylating tyrosine residues (6, 9, 10). On the other hand, a similar cellular protein kinase, pp60$^{c\text{-}src}$, has been identified in normal cells. The amount of pp60$^{v\text{-}src}$ present in RSV-transformed cells seems to be at least 100 times the amount of pp60$^{c\text{-}src}$ present in uninfected cells (11–14). It is, therefore, possible that viral transformation may result from unscheduled phosphorylation of cellular proteins by pp60$^{v\text{-}src}$.

Recently, various possible cellular target proteins of pp60$^{v\text{-}src}$ have been identified. A 36-kDal protein (15–19) is phosphorylated in cells infected with temperature-sensitive transformation mutants of RSV within 1 hr after a shift from the nonpermissive to the permissive temperature (15, 19). Similarly, vinculin, a cytoskeletal protein with a molecular mass of 130 kDal, is phosphorylated on transformation by RSV (20). In addition, a protein with a molecular mass of 50 kDal was found in immunoprecipitates of pp60$^{v\text{-}src}$, suggesting the possibility that this may also be a target of pp60$^{v\text{-}src}$ (21–23). Previously, phosphorylation of the 36-kDal protein of RSV-transformed cells has been reported from this laboratory both *in vivo* (19) and *in vitro* (16). In this paper, we report intracellular localization of the phosphorylated 36-kDal protein in plasma membrane and, using our *in vitro* system, we show that the membrane fraction alone can phosphorylate the 36-kDal protein.

MATERIALS AND METHODS

Cells and Viruses. Chicken embryo fibroblasts used in these experiments were derived from 11-day SPAFAS (specific pathogen-free avian supply) embryos and grown in Ham's F-10 medium/5% calf serum/1% chicken serum (24). Cells were infected with the Schmidt–Ruppin strain of RSV, SR-A (25), and were cultured at 37°C.

Labeling the Cells *in Vivo*. Cells transformed by SR-RSV-A were grown to confluency in culture and labeled with $^{32}PO_4$ (New England Nuclear; carrier free) at 0.5–1 mCi/ml (1 Ci = 37 GBq) for 2.5 hr at 36°C in phosphate-free medium. Cells were washed and lysed in lysis buffer (26) by repeated freezing and thawing.

Subcellular Fractionation of RSV-Transformed Chicken Embryo Fibroblasts. ^{32}P-Labeled cells (2×10^7) were fractionated as described by Hay (27) with modifications. Cells were washed two or three times with phosphate-buffered saline (140 mM NaCl/1.8 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$, pH 7.4) and homogenized in 5 mM KCl/1 mM $MgCl_2$/20 mM Hepes, pH 7.1, with a Dounce homogenizer. The homogenate was centrifuged at $1,000 \times g$ for 5 min to remove nuclei, and the supernatant fluid was centrifuged at $100,000 \times g$ for 30 min. The resulting supernatant was designated S100 and was used as the cytoplasmic fraction, and the pellet thus formed was designated P100 and was used as the crude membrane fraction. Fraction S100 was dialyzed against a solution containing 1 mM phenylmethylsulfonyl fluoride (as a protease inhibitor) and lyophilized and then dissolved in O'Farrell's lysis buffer and subjected to two-dimensional electrophoresis (26). The crude membrane and nuclear fractions were examined for the presence of pp36 in a similar fashion.

Nuclei Preparation. In most of the experiments, the nuclear fraction was prepared as described above. To obtain purified nuclei, an alternative method was used (L. Cohen, personal communication). Transformed fibroblasts (10^7 cells) were labeled with $^{32}P_i$ as described above, washed twice with 0.02 M Tris·HCl, pH 7.5/0.15 M NaCl and scraped from the plates. The cells were then washed twice with 10 mM Tris/K maleate, pH 7.4/0.15 M NaCl. The cell pellet was suspended in 5 ml

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Abbreviations: RSV, Rous sarcoma virus; kDal, kilodalton(s); pp36, phosphoprotein of 36 kDal; SR-RSV, Schmidt–Ruppin strain of RSV; INT, 2-(4-indophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium.

of a buffer (10 mM Tris/K maleate, pH 7.4/10 mM glycine/5 mM $MgCl_2$ containing Trasylol at 100 units/ml and 0.1 mM each of *N*-α-*p*-tosyl-L-lysine chloromethyl ketone·HCl and L-1-tosylamido-2-phenylethyl chloromethyl ketone as protease inhibitors. The cells were then homogenized by 40 strokes with the Dounce B pestle. The homogenate (5 ml) was overlaid on a Percoll discontinuous gradient (5 ml of 19% Percoll, 7 ml of 16% Percoll, 5 ml of 11% Percoll containing 0.5% Triton X-100, and 5 ml of 7% Percoll). The gradient was centrifuged at 365 × *g* for 5 min and then at 4,000 × *g* for 5 min. The pellet thus formed was regarded as purified nuclei. Microscopic observation showed almost no contamination with cell debris. Two nuclei preparations as described above gave similar results regarding the localization of pp36.

Two-Dimensional Gel Electrophoresis and Autoradiography. Two-dimensional gel electrophoresis was carried out according to the method of O'Farrell (26). Approximately 2.5–10 × 10^5 cpm of trichloroacetic acid precipitable material was applied on the first dimension gel and fractionated by nonequilibrium pH gradient electrophoresis at pH 3–10. The second dimension was $NaDodSO_4$ gel electrophoresis. Gels were stained with Coomassie blue, dried, and exposed to Kodak X-Omat-R film at −70°C with the aid of Dupont intensifying screen. The following proteins were used as markers: β-galactosidase (130 kDal), bovine serum albumin (68 kDal), ovalbumin (43 kDal), and cytochrome *c* (11.7 kDal).

Subcellular Fractionation of Crude Membrane. Fractionation of the crude membrane was done according to the discontinuous sucrose gradient fractionation method described by Courtneidge *et al.* (28). After fractionation, each interface was centrifuged separately and the pellets were dissolved in lysis buffer for two-dimensional analysis.

The purity of the subcellular fractions was evaluated as described (27, 29), from assays of Na^+,K^+-ATPase (plasma membrane), NADH diaphorase (endoplasmic reticulum), and succinic 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium (INT) reductase (mitochondria). Protein content of fractions was determined by the Lowry procedure (30) with bovine serum albumin as the standard.

Cell-Free Phosphorylation of Proteins from SR-RSV-Infected Chicken Embryo Fibroblasts. Cells were washed with 0.02 M Tris·HCl, pH 7.5/0.15 M NaCl, scraped, suspended in RIPA buffer (150 mM NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% $NaDodSO_4$/50 mM Tris·HCl, pH 7.2)/1% Trasylol (31), incubated at 0°C for 30 min, and centrifuged for 30 min at 15,000 × *g*. The reaction mixture for the kinase assay (32) was similar to that reported previously (16), 50 mM Tris·HCl, pH 7.2/10 mM $MgCl_2$/10 mM NaF/30 μM EGTA/25 μM cAMP containing 30 μCi of [γ-^{32}P]ATP (2,000 Ci/mmol) and 40 μl of cell extract (100–150 μg of protein) in a total volume of 100 μl. The reaction mixture was incubated at 30°C. After 30 min, the reaction was stopped by adding 0.5 ml of cold (0°C) 10% trichloroacetic acid, and the radioactive precipitate was subjected to gel electrophoresis. For assay of phosphorylation of the 36-kDal protein in cytoplasm, fraction S100 (3 ml, 5.75 mg of protein) was dialyzed overnight against 1 mM phenylmethylsulfonyl fluoride, lyophilized, and suspended in 0.3 ml of RIPA buffer; 40 μl of the suspension was incubated with [γ-^{32}P]ATP as above. Alternatively, 0.3 ml of fraction S100 was mixed with concentrated RIPA buffer to obtain the final concentration as above and 40 μl of this suspension was incubated with [γ-^{32}P]ATP. *In vitro* phosphorylation of the isolated membrane fraction was carried out under conditions identical to those used for phosphorylation of the cytoplasmic fraction.

RESULTS

Membrane Localization of pp36 Labeled *in Vivo* with $^{32}PO_4$. Chicken embryo fibroblasts transformed with SR-RSV were labeled *in vivo* with $^{32}PO_4$, and the total cellular phosphoproteins were analyzed by nonequilibrium pH gradient electrophoresis (Fig. 1*A*). As shown, the 36-kDal protein was phosphorylated. For comparison, normal uninfected cells were labeled and analyzed under the same conditions, and pp36 was shown to be absent (data not shown). These observations confirm the conclusion previously reached by our laboratory (16, 19) as well as by others (15, 17, 18) that the appearance of pp36 is transformation dependent. In an attempt to localize this protein in subcellular fractions, nuclear, cytosol (S100), and crude membrane (P100) fractions were prepared, and the phosphoproteins present in each fraction were analyzed by two-dimensional gel electrophoresis. Fig. 1 shows that pp36 is exclusively found in the

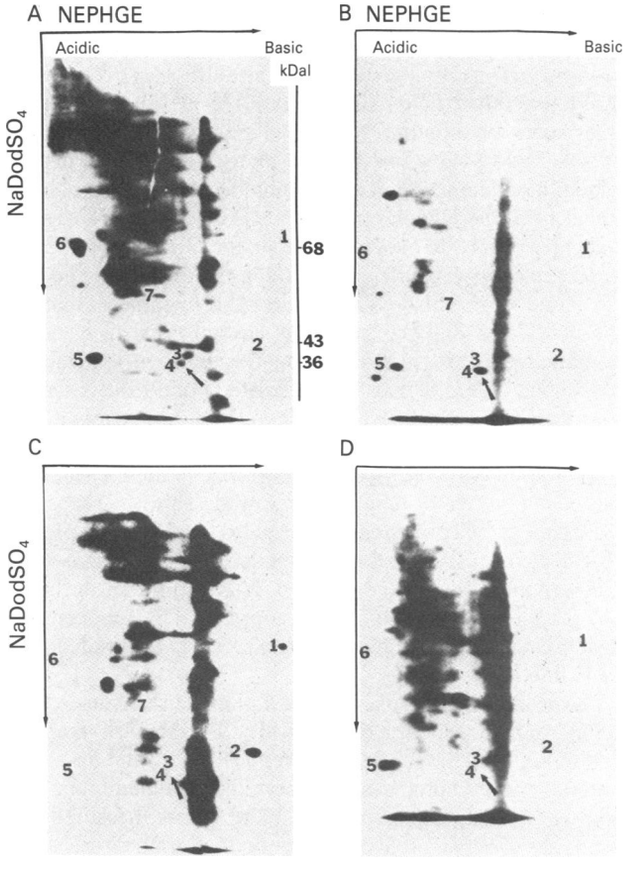

FIG. 1. Association of the phosphorylated 36-kDal protein with the crude membrane fraction of SR-RSV-A-transformed chicken embryo fibroblasts. (*A*) Two-dimensional analysis of total phosphoproteins from SR-RSV-transformed chicken embryo fibroblasts. SR-RSV-transformed chicken embryo fibroblasts were labeled with $^{32}PO_4$ at 0.5 mCi/ml for 2.5 hr in phosphate-free medium. Cells were scraped and lysed in O'Farrell's lysis buffer and the phosphoprotein-containing lysate (300,000 cpm) was separated by nonequilibrium pH gradient electrophoresis (NEPHGE) using pH 3–10 Ampholine in the first dimension and $NaDodSO_4$/polyacrylamide gel electrophoresis in the second dimension. The arrow indicates the transformation-specific phosphorylated 36-kDal protein (spot 4); other radioactive spots are numbered for comparison. (*B*) The membrane fraction was prepared from ^{32}P-labeled transformed chicken embryo fibroblasts as described above and analyzed as in *A*. The arrow indicates the spot corresponding to the 36-kDal phosphoprotein. (*C*) The nuclear fraction was analyzed as in *A*. The arrow indicates that the 36-kDal phosphoprotein is absent. (*D*) The cytoplasmic fraction was obtained in parallel with the membrane fraction as in *B*. The arrow indicates that the 36-kDal phosphoprotein is absent from this fraction.

Table 1. Comparison of seven radioactive spots from two-dimensional gels of subcellular fractions

Spot		cpm			
		Cell	Nuclei	Membrane	Cytoplasm
1		48	124	12	10
2		58	184	22	12
3		203	14	20	164
4	(36 kDal)	134	12	524	14
5		684	19	624	504
6		744	14	144	24
7		110	17	17	287

Radioactive spots 1–7 in Fig. 1 were excised from four gels (300,000 cpm was applied to each gel), and their radioactivities were determined. Results given are values obtained after subtraction of the gel background.

crude membrane lysate (Fig. 1*B*) and cannot be detected in either the nuclear or the cytoplasmic fractions (Fig. 1 *C* and *D*). The 36-kDal phosphoprotein from the total cell and subcellular fractions was quantitated by cutting the pp36 spots from the gels and determining their radioactivity (Table 1). In addition, six other spots were randomly selected and quantitated. The results in Table 1 show that the phosphorylated 36-kDal protein is found predominantly in the membrane fraction and is almost absent from the cytoplasmic and nuclear fractions. There are some other spots that are found predominantly in one fraction; for example, spots 3 and 7 are found in the cytoplasm and spot 1 is found in the nuclei. To confirm the hypothesis that the phosphorylated 36-kDal protein is located exclusively in the membrane fraction, the distribution of radioactive 36-kDal protein among these fractions was calculated (Table 2). As shown, more than 80% of the total pp36 is found in the crude membrane fraction. Also, almost complete recovery of phosphorylated 36-kDal protein was achieved and almost 90% of the total acid-insoluble radioactive protein was recovered. This is perhaps due to a higher stability of tyrosine-phosphorylated 36-kDal protein (16–18) compared with other phosphoproteins. The ratio of the radioactivity of the phosphorylated 36-kDal protein to that of total phosphoproteins indicates an approximately 4-fold enrichment of the phosphorylated 36-kDal protein in the crude membrane fraction.

Localization of Phosphoproteins 1 and 7 to the Nuclear and Cytoplasmic Fractions, Respectively. To establish that the method we used for subcellular fractionation is valid for all the proteins that are not necessarily located in the membrane, the radioactivities of phosphoproteins 1 and 7 were measured; the results are given in Table 3. Both of these phosphoproteins are fairly stable, as shown by their more than 80% recovery in subcellular fractions. An approximately 3-fold enrichment of these phosphoproteins was observed in the nuclear and cytoplasmic fractions, respectively. We therefore conclude that the enrichment of pp36 in the membrane fraction as discussed above is not due to the presence of general protein phosphatase in nuclear and cytoplasmic fractions.

Table 2. Distribution of pp36 among subcellular components

	Total extract	Membrane	Cytoplasm	Nuclei
Total cpm of pp36 recovered (a)	3,899	3,422	109	129
% pp36 in fraction	100	87.8	2.8	3.3
Total cpm recovered in fraction (b)	34,929,500	7,837,500	11,012,200	10,954,400
(a/b) × 10^4	1.11	4.36	0.09	0.11

pp36 radioactivity was determined by scintillation counting.

Plasma Membrane Association of pp36. The membrane fraction described above contains not only plasma membranes but also endoplasmic reticulum, lysosomes, Golgi apparatus, and such (27). For further localization of the phosphorylated 36-kDal protein, the crude membrane fraction was further fractionated by the discontinuous sucrose density centrifugation method of Hay (27). In this procedure, relatively pure membranes are recovered at the interface of the discontinuous sucrose gradient. The purity of the membrane fractions was supported by the distribution of specific enzyme markers such as Na^+,K^+-ATPase (plasma membrane), NADH diaphorase (endoplasmic reticulum), and succinic INT reductase (mitochondria). As shown in Table 4, the major portion of the Na^+,K^+-ATPase was recovered in the plasma membrane fraction (20–35% sucrose). The specific activity of this enzyme was also the highest in this fraction. Very little (11%) of the total activity of NADH diaphorase was recovered in the plasma membrane fraction, indicating that contamination of the plasma membrane fraction with endoplasmic reticulum is minimal. Furthermore, the activity of succinic INT reductase was highest in the fraction containing lysosomes, mitochondria, and Golgi apparatus (40–50% sucrose). Less than 20% of succinic INT reductase activity was found in the plasma membrane, suggesting very little contamination by lysosomes and such in the plasma membrane fraction (data not shown). Judging from the distribution of these enzymes, the purity of our membrane fractions is comparable with the previously published values (28) for purified membranes from fibroblasts. As shown in Table 5, the major portion (more than 50%) of the phosphorylated 36-kDal protein was recovered in the plasma membrane fraction. Less than 30% and less than 20% of pp36 was found in the endoplasmic reticulum fraction and the fraction containing lysosomes and such respectively.

***In Vitro* Phosphorylation of the 36-kDal Protein in the Purified Membrane Preparation with [γ-^{32}P]ATP.** A cell-free system in which the 36-kDal protein can be phosphorylated has been described (16). Because the 60-kDal *src* protein is a membrane protein (28, 33, 34), phosphorylation of the 36-kDal protein by the membrane fraction will confirm the hypothesis that

Table 3. Localization of phosphoproteins 1 and 7 to the nuclear and cytoplasmic fractions, respectively

	Spot 1		Spot 7		(Spot cpm/total cpm applied) × 10^4	
	cpm	% total	cpm	% total	Spot 1	Spot 7
Total extract	1,396	100	3,201	100	0.39	0.91
Membrane	78	5.8	111	3.4	0.09	0.14
Cytoplasm	91	6.5	2,633	82.2	0.08	2.39
Nuclei	1,131	85.0	155	4.8	1.03	0.14

Results represent the sum of four gels. In each case, 300,000 cpm was applied to each gel. The spots were cut out from the gels, and their radioactivities were determined measured in a scintillation counter.

Table 4. Distribution of protein and Na^+,K^+-ATPase and NADH diaphorase activities among membrane fractions

Fraction	Protein, %	NADH diaphorase Activity, (μmol/mg)/min	NADH diaphorase % total recovery	Na^+,K^+-ATPase P_i, (μmol/mg)/hr	Na^+,K^+-ATPase % total recovery
20–35% sucrose	24.5	0.192	11.0	5.4	60.0
35–40% sucrose	30.5	0.539	38.5	2.1	29.0
40–50% sucrose	45.0	0.479	50.5	0.54	11.0

SR-RSV-transformed fibroblasts were disrupted and fractionated. Enzymatic activities were measured as described (27, 29).

the 36-kDal protein resides in the membrane. For this purpose, the plasma membrane fraction was incubated with [γ-^{32}P]ATP and the phosphoproteins were analyzed by two-dimensional gel electrophoresis. As shown in Fig. 2*B*, the plasma membrane fraction phosphorylated the 36-kDal protein. For comparison, a crude whole cell extract was incubated with [γ-^{32}P]ATP in parallel. In confirmation of the previous findings, the 36-kDal protein was phosphorylated *in vitro* (Fig. 2*A*). The number of phosphorylated radioactive spots was less with the membrane system than with the whole cell extract.

Because of the report that pp60^{v-src} is present in cytoplasm (35), we examined the possible *in vitro* phosphorylation of the 36-kDal protein in the cytoplasmic fraction. Two different preparations of cytoplasm were tested. Very little phosphorylation of proteins was observed with these preparations under conditions in which the crude membrane fraction actively phosphorylated proteins (data not shown). The small amount of ^{32}P-labeled macromolecules obtained with the cytoplasm was further analyzed for the presence of pp36 by two-dimensional gel electrophoresis. No pp36 was detected (data not shown). These observations further support the conclusion that pp36 is localized to the membranes.

DISCUSSION

It has previously been shown in our laboratory that a 36-kDal protein is phosphorylated in myotube cultures infected with RSV (19) and that this protein is phosphorylated *in vitro* with [γ-^{32}P]ATP in a crude extract from RSV-infected chicken fibroblasts (16). Studies in other laboratories in which *in vivo* labeling (15, 18) and *in vitro*-reconstituted systems (17) were used have identified a similar protein as a target protein of *src* kinase. The

Table 5. Distribution of pp36 in three membrane fractions

	Sucrose 20–35%	Sucrose 35–40%	Sucrose 40–50%
Total radioactivity recovered in interfaces, cpm	1,075,400	836,680	1,799,200
Total pp36 in fraction,* arbitrary units	1.15	0.64	0.42
% total pp36 recovered	52	29	19
Membranous element(s)	Plasma membrane	Endoplasmic reticulum	Lysosomes and such

* ^{32}P-Labeled pp36 was determined by densitometric scanning of the autoradiograms. These values, together with the total radioactivity recovered in each fraction, were used to calculate total pp36 in each fraction.

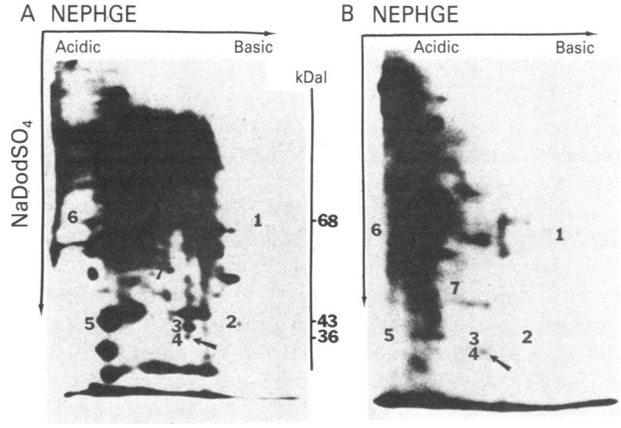

FIG. 2. *In vitro* phosphorylation of the 36-kDal protein in a crude extract and the membrane fraction of SR-RSV-transformed chicken embryo fibroblasts. (*A*) A crude extract of SR-RSV-A-transformed chicken embryo fibroblasts (100–150 μg of protein) was incubated with [γ-^{32}P]ATP, and 250,000 cpm of product was subjected to two-dimensional analysis. The arrow indicates the phosphorylated 36-kDal protein. (*B*) The membrane fraction from the transformed cells (50 μg) was incubated with [γ-^{32}P]ATP, and 250,000 cpm of product was analyzed as in *A*. The arrow indicates the *in vitro*-phosphorylated 36-kDal protein.

phosphorylated 36-kDal protein that we have identified is probably identical to the 36-kDal protein reported by Radke and Martin (15, 18) on the following bases. (*i*) Phosphorylation of this protein is temperature sensitive in cells infected with temperature-sensitive RSV transformation mutants (15, 19). (*ii*) This protein is phosphorylated at the tyrosine residue (16, 18). (*iii*) Our 36-kDal protein behaves identically to that reported by Radke and Martin in two-dimensional gel electrophoresis (15, 16, 19). (*iv*) The partial proteolytic digestion patterns of our ^{35}S- and ^{32}P-labeled 36-kDal protein are identical to those reported by Radke and Martin (18) (our data will be published elsewhere). These two 36-kDal proteins are probably similar or identical to the 34-kDal (17, 36), 38-kDal (37), and 39-kDal (38) proteins reported by others. However, further studies are needed to definitely establish the identity of these proteins.

In the study reported here, we attempted to determine the cellular location of the phosphorylated 36-kDal protein. The phosphorylated 36-kDal protein cannot be localized by the conventional immunofluorescence method using cytological observation because antibody against pp36 would react against both nonphosphorylated and phosphorylated protein. The only available method is to fractionate the subcellular components and determine the pp36 in each fraction. The data presented in this communication are consistent with the hypothesis that pp36 is associated with the plasma membrane. First, more than 80% of the total pp36 was recovered in the crude membrane fraction. Second, the major portion of recovered pp36 (*in vivo* phosphorylated) of the membrane was found in the plasma membrane fraction. On the basis of similar percentage distribution data, Courtneidge *et al.* (28) concluded that pp60^{v-src} is associated with the plasma membrane. Third, incubation of the purified membrane fraction with [γ-^{32}P]ATP resulted in phosphorylation of the 36-kDal protein. Fourth, preliminary evidence suggests that nonphosphorylated 36-kDal protein is also found in the membrane fraction (unpublished data). The hypothesis that pp36 is associated with the plasma membrane is consistent with the observation by Cheng and Chen (36) that this protein is found in the fraction containing cytoskeletal and integral membrane proteins.

Protein phosphorylation has been found to regulate a large

number of cellular processes (39, 40). The possibility that phosphorylation of membrane proteins by membrane-bound kinases is involved in functional regulation has been suggested (41–43). It is, therefore, conceivable that phosphorylation of the membrane-associated 36-kDal protein by $pp60^{v-src}$ may play a key role in structural and functional changes of membranes on transformation (44–46).

As for the function of the 36-kDal protein, no definite information is available at present. A cellular protein with a molecular mass of 38 kDal, perhaps closely related to the 36-kDal protein, has recently been reported to be phosphorylated on transformation by RSV (37). This protein was identified as a subunit of malic dehydrogenase. However, functional change of this enzyme by phosphorylation has not been reported. Further studies are necessary to establish the enzymatic or structural function of the 36-kDal protein.

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