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Membrane Association of the Transforming Protein of Avian Sarcoma Virus UR2 and Mutants Temperature Sensitive for Cellular Transformation and Protein Kinase Activity

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The localization of the transforming protein P68$^{gag\text{-}ros}$ of avian sarcoma virus UR2, which has a hydrophobic region at the N terminus of its *ros*-specific tyrosine kinase-encoding sequence, was examined by subcellular fractionation. P68 behaved as an integral membrane protein associated with the plasma membrane of transformed cells. P68 became membrane associated very rapidly in its biogenesis. Three temperature-sensitive mutants of UR2 were isolated and characterized. Cells infected with the mutants were temperature sensitive for morphological alteration and colony formation. The mutant P68 proteins were membrane associated in mutant-infected cells regardless of the temperature but were active as protein kinases only at the permissive temperature. The results suggest that P68 is a membrane-associated protein whose kinase activity plays a crucial role in UR2-mediated cell transformation.

Avian sarcoma virus UR2 is a recently characterized replication-defective virus which induces sarcomas in vivo (4) and efficiently transforms chicken embryo fibroblasts (CEF) in vitro to a distinctive elongated morphology retaining a high level of cytoskeletal organization (3, 4, 34). The UR2 transforming protein, P68$^{gag\text{-}ros}$, which contains fused p19 and v-*ros* sequences, is a member of the tyrosine kinase oncogene family (32, 33, 52). The P68 v-*ros*-specific sequence contains distinctive hydrophobic regions and unique amino acid changes and insertions within the conserved kinase domain, suggesting that P68 may have a unique target specificity (33). The kinase domain of P68 shares amino acid sequence homology with the intracellular domains of the human epidermal growth factor receptor and insulin receptor (17, 18, 50, 51). This structural homology, and the presence of a hydrophobic region at the N terminus of the *ros*-specific sequence, suggests that P68 is a transmembrane protein with a cytoplasmic tyrosine kinase domain. However, indirect immunofluorescence microscopy on acetone-fixed, permeabilized, UR2-transformed cells failed to assign a definite location to the transforming protein, except to rule out a nuclear localization (34).

In this paper, we describe the plasma membrane localization of P68 in UR2-transformed cells by subcellular fractionation and characterize differences between its membrane association and that of p60^{src}, the membrane-associated tyrosine kinase transforming protein (see reference 27 for a review) of Rous sarcoma virus (RSV). We also describe the isolation and characterization of three mutants of UR2 that are temperature sensitive (*ts*) for cellular transformation. P68 is membrane associated in the *ts*-mutant-infected cells at both the permissive and nonpermissive temperatures, but the P68 protein kinase activity is greatly reduced at the nonpermissive temperature.

MATERIALS AND METHODS

Cell culture and viruses. Cultures of secondary CEF were maintained and infected as described previously (4, 52). The isolation of avian sarcoma virus UR2 and its helper UR2-associated virus (UR2AV) and the isolation of UR2 and UR2AV viruses by transfection of CEF with molecularly cloned UR2 and UR2AV DNAs have been described (4, 32, 33). Unless stated otherwise, virus from transfection was used as wild-type (wt) UR2 (UR2AV) virus for experiments; the cloned DNAs are biologically active and indistinguishable in effect upon CEF from the parental viruses (32, 33). The Schmidt-Ruppin subgroup A strain of RSV was also used.

Isolation of *ts* mutants. To mutagenize the virus, CEF fully transformed with a wt UR2 stock (not from molecular clones but from the original UR2 stock [4]) derived from a single focus were treated with 25 μg of 5-azacytidine per ml for 24 h. Virus obtained from the treated cultures was used for colony formation in soft agar. Colonies were isolated and cultured together with fresh chicken cells in the presence of UR2AV to facilitate UR2 production.

For all experiments with *ts* mutants, CEF were infected at 37°C, and after cultures were transformed, the infected cells were subcultured and incubated at 37 or 42°C for at least 2 days before use in experiments.

Labeling conditions. For [^{3}H]leucine, [^{35}S]methionine, or $^{32}P_i$ labeling, cultures were preincubated for 2 h in minimal essential medium lacking leucine, methionine, or phosphate, respectively, and supplemented with 5% dialyzed calf serum before being labeled for the indicated amount of time. For pulse-chase analysis, labeled cultures were washed and incubated in the following complete medium: Ham F10 medium containing 10% tryptose phosphate broth, 5% calf serum, 1% chick serum, and 1% dimethyl sulfoxide. For labeling with [^{3}H]myristic acid or [^{3}H]palmitic acid, labeled fatty acid was dried under nitrogen, dissolved in a minimum volume of dimethyl sulfoxide, and added to cultures in complete medium. For labeling with [^{3}H]glucosamine, label was added to cultures in complete medium.

Immunoprecipitation and kinase assay. Cell extracts were prepared in RIPA buffer containing 10 mM Tris-hydrochloride (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, and 100 kallikrein inactivating units of aprotinin (Trasylol; FBA Pharamaceuticals, Inc.) per ml. For experiments with *ts*

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mutants, RIPA buffer lacking SDS was used to prevent excessive denaturation of kinase activity. Extracts were immunoprecipitated with saturating amounts of antiserum as described previously (19, 20). A serum from a rabbit bearing a tumor induced by Schmidt-Ruppin subgroup D RSV (TBR serum) was used for immunoprecipitation (9). The TBR serum used had a high titer against *gag* proteins and gPr92^{env} and did not cross-react with c-*src* protein. Immunoprecipitates were washed three times with RIPA buffer containing 300 mM NaCl, then two times with RIPA buffer containing 10 mM NaCl (low-salt RIPA). For assay of protein kinase activity, RIPA-washed immunoprecipitates were washed twice with kinase buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] [pH 7.4]–10 mM $MnCl_2$) and resuspended in kinase buffer with 0.1 μM [γ-^{32}P]ATP. The reaction proceeded for 15 min at 25°C and then was terminated by washing three times in low-salt RIPA.

For assay of the phosphorylation of the exogenous substrate enolase, rabbit muscle enolase (Sigma Chemical Co.) was acid denatured for 5 min at 30°C as described previously (11), and then 5 μg was added to each kinase reaction as above. After a 15-min incubation at 25°C, immune complexes were washed three times with low-salt RIPA. Under these conditions, there was no change in P68 phosphorylation upon inclusion of enolase in the reaction mixture, and as described previously for enolase phosphorylation reactions with immune complexes containing other viral tyrosine kinases (11), the phosphorylated enolase remained bound to the immune complexes. Immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels.

To measure heat inactivation of kinase activity, lysates of infected cells maintained at 37°C were prepared, and portions were incubated at 41°C for the specified time. Heat inactivation was stopped by the addition of cold lysis buffer, and samples were subjected to immunoprecipitation and kinase assay.

Gels containing ^{3}H- or ^{35}S-labeled proteins were fluorographed with the water-soluble fluor Amplify (Amersham Corp.). Gels containing ^{32}P-labeled proteins were dried without fluorography. Where specified, to facilitate detection of phosphotyrosine-containing phosphoproteins labeled in vivo, gels were incubated in alkali as described previously (12).

Cell fractionation and gradient analysis. Cells were fractionated by differential centrifugation into particulate and cytosolic fractions as described previously (16, 36, 37). Crude membrane fractions were floated on discontinuous sucrose gradients as described previously (36). Fractionation of cell extracts on glycerol gradients for analysis of the sedimentation behavior of proteins was as described previously (8).

RESULTS

Characterization of P68 in UR2-transformed cells. Extracts from [^{3}H]leucine-labeled uninfected cells, UR2AV-infected cells, and UR2-transformed cells were immunoprecipitated with TBR serum. This particular TBR serum had a high titer against viral structural proteins and immunoprecipitated P68, since P68 is a fusion protein containing *gag* antigenic determinants at its N terminus (32, 33). P68 was specifically immunoprecipitated from UR2-transformed cells (Fig. 1). As observed previously (20, 32), TBR serum immunoprecipitates containing P68 catalyzed the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP to tyrosine residues in P68 and, to a lesser extent, the heavy chain of immunoglobulin G; no kinase activity was detectable in uninfected cells or UR2AV-infected cells (data not shown).

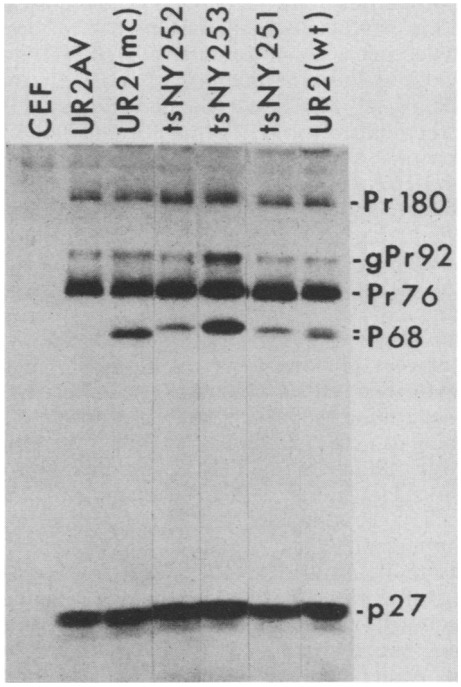

FIG. 1. Immunoprecipitation of P68$^{gag\text{-}ros}$ by TBR serum. Cultures maintained at 37°C were labeled for 4 h with [^{3}H]leucine and lysed in RIPA buffer, and lysates were immunoprecipitated with TBR serum. Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. The positions of viral structural proteins Pr180$^{gag\text{-}pol}$, gPr92^{env}, Pr76^{gag}, and p27^{gag} and transforming protein P68$^{gag\text{-}ros}$ are indicated. Note the P68 doublet in the wt-UR2-transformed cells; the lower band comigrates with the single P68 species seen in cells infected with molecularly cloned UR2 (UR2AV) [UR2(mc)], while the upper band comigrates with the single P68 species seen in *ts* mutant virus-infected cells.

A P68 doublet was immunoprecipitated from cells transformed by wt UR2 (Fig. 1). This doublet has been observed previously (20, 32). The relative proportions of the two P68 species differed in cells infected with UR2 (UR2AV) stocks with different passage histories (data not shown), although the reason for this variation is unknown. A single P68 species, comigrating with the lower band, was seen in immunoprecipitates prepared from cells infected with virus derived from transfection with molecularly cloned UR2 and UR2AV DNAs (Fig. 1). A single P68 species, comigrating with the upper band, was seen in cells infected with the UR2 *ts* mutants, whose isolation and characterization will be discussed in detail in a later section. The *ts* mutants were obtained from a stock derived from a single focus. Upon phosphorylation in an in vitro kinase assay, no mobility differences could be resolved; all P68 species comigrated as a single species whose electrophoretic mobility appeared to be the same as that of the upper metabolically labeled P68 species (data not shown). The *Staphylococcus aureus* V8 protease maps of the metabolically labeled and in vitro-phosphorylated P68s were indistinguishable (data not shown). Both P68 species are phosphoproteins in vivo, with indistinguishable V8 protease maps (data not shown). Apparently, there are variant forms of P68, inducing indistinguishable transformed phenotypes and, other than the minor electrophoretic mobility difference, indistinguishable bio-

chemical properties (data not shown). The precise reason for the variation has yet to be determined. The virus derived from transfection with molecularly cloned DNAs was used as wt UR2 for all the experiments whose descriptions follow.

No labeling of P68 could be detected in TBR serum immunoprecipitates from UR2-transformed cells labeled for 4 or 16 h with [^{3}H]myristic acid or [^{3}H]palmitic acid (conditions in which labeling of p60^{src} in RSV-transformed cells was easily detected; data not shown), suggesting that P68 is not modified with fatty acid as are the transforming proteins of RSV, Abelson murine leukemia virus, and Harvey sarcoma virus (42, 46). No labeling of P68 could be detected in TBR serum immunoprecipitates from UR2-transformed cells labeled for 4 h with [^{3}H]glucosamine (conditions in which labeling of *env* glycoproteins gPr92, gp85, and gp37 in UR2-transformed cells was readily detected; data not shown), suggesting that P68 is not a glycoprotein as are the transforming proteins of avian erythroblastosis virus (24, 38) and the McDonough strain of feline sarcoma virus (1, 2, 40).

Subcellular localization of P68. To examine the localization of P68, [^{3}H]leucine-labeled UR2-transformed cells were Dounce homogenized and separated into nuclear (P1), crude membrane (P100), and cytosolic (S100) fractions by differential centrifugation. P68 was immunoprecipitated from these fractions, and its distribution was quantitated by determining the ^{3}H radioactivity associated with the P68 protein gel band or the ^{32}P radioactivity associated with the phosphorylated P68 band after in vitro kinase assay of the immunoprecipitates (data not shown). These methods of quantitation produced congruent results: 17% of the total P68 was in the P1 fraction, 61% was in the P100 fraction, and 22% was in the S100 fraction (average of three independent fractionations). Based upon marker enzyme assay, the P1 fraction from Dounce-homogenized CEF was somewhat contaminated with plasma membrane, endoplasmic reticulum, and unbroken cells; and the cytosolic fraction was also contaminated with plasma membranes and membranous organelles (data not shown; reference 27), so the results suggest that the majority of intracellular P68 was membrane associated. The membrane association of P68 was not salt sensitive over a concentration range of 10 to 300 mM NaCl (data not shown), unlike the salt-sensitive membrane association of the transforming protein of Fujinami sarcoma virus (19, 36).

The crude membrane fraction was further fractionated by equilibrium centrifugation in a discontinuous sucrose gradient (Table 1); the P68 kinase activity was most highly enriched in the light (plasma) membrane fraction. The distribution of [^{3}H]leucine-labeled P68 (data not shown) paralleled the distribution of kinase activity, suggesting that P68 is a plasma membrane-associated protein, although there was a significant amount of P68 that cofractionated with intermediate and heavy membrane fractions (Table 1).

TABLE 1. Fractionation of membrane-bound P68 kinase activity on a discontinuous sucrose gradient[a]

Fraction (%)	%P68	Sp act
20–35	49	6.0
35–40	22	1.7
40–50	29	1.0

[a] UR2-transformed cells were labeled for 4 h with [^{3}H]leucine and Dounce homogenized, and a crude membrane pellet was prepared and fractionated on a discontinuous sucrose gradient. Membrane fractions were analyzed for P68 kinase activity. The 20–35% interface fraction is enriched for plasma membranes, and the 40–50% interface fraction is enriched for rough endoplasmic reticulum, Golgi, and mitochondrial membranes (15, 27). Cumulative recovery of P68 is normalized to 100%, and specific activities are normalized to the specific activity of the P68 kinase activity found in the 40–50% interface. Specific activity is defined as the ^{32}P counts per minute in P68 in a kinase assay divided by the total amount of protein in the fraction as indicated by trichloroacetic acid-precipitable [^{3}H]leucine counts per minute. Values shown represent the average of three separate determinations.

TABLE 2. Extraction of proteins from crude membranes from UR2- or RSV-transformed cells

Extraction	%Protein in pellet after extraction[a]:		
	P68 kinase	gPr92^{env} glycoprotein[b]	p60^{src} kinase[c]
Control	95	98	94
600 mM NaCl	64	ND[d]	66
10 mM EDTA	90	97	92
10 mM EGTA[e]	88	95	94
1% Triton	41	39	48
1% Deoxycholate	27	20	30

[a] Crude membrane fractions from transformed cells were suspended by Dounce homogenization in 10 mM Tris-hydrochloride (pH 7.4)–100 mM NaCl (except in the case of 600 mM NaCl extraction) with the indicated reagent, incubated for 15 min at 0°C, and then separated into pellet and supernatant fractions by centrifugation (100,000 × *g*) for 30 min. Fractions were solubilized in RIPA buffer and immunoprecipitated with TBR serum. Immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels. Data are normalized to 100% cumulative recovery.

[b] UR2-transformed cells were labeled with [^{3}H]glucosamine for 4 h before fractionation. gPr92^{env} bands immunoprecipitated by TBR serum were excised from gels, and incorporated ^{3}H cpm was quantitated.

[c] RSV (Schmidt-Ruppin subgroup A)-transformed cells were fractionated. Phosphorylation of TBR immunoglobulin G was quantitated by kinase assay.

[d] ND, Not determined.

[e] EGTA, Ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid.

P68 is an integral membrane protein. To determine whether the membrane association of P68 was specific or due to a loose association with membranes as a peripheral protein, the ability of a variety of reagents to solubilize P68 from crude membranes was tested (Table 2). The behavior of two different membrane-associated proteins in transformed CEF that act as integral membrane proteins was monitored in parallel; p60^{src}, whose membrane association in RSV-transformed CEF is mediated by covalently bound myristic acid (16, 42), and gPr92^{env}, which has a transmembrane hydrophobic domain serving as a membrane anchor (25, 43), were assayed (Table 2). Like p60^{src} and gPr92^{env}, P68 was not significantly solubilized by high salt or divalent cation chelators but was only solubilized by detergents. These results suggest that P68 is an integral membrane protein.

Transport of P68 to the membrane. Pulse-chase labeling and in vitro translation has suggested that P68 is translated directly to a 68-kilodalton form that does not undergo any proteolytic cleavage during its lifetime (20). To determine how rapidly P68 became membrane associated, UR2-transformed cells were pulse-labeled with [^{35}S]methionine for 10 min, then chased with complete medium for 0, 5, 10, 15, and 30 min, and then separated into membrane and cytosolic fractions. After the 10-min labeling, even without any chase, P68 was predominantly membrane associated (data not shown). UR2-transformed cells were pulse-labeled with [^{35}S]methionine for shorter times and fractionated (Fig. 2). P68 was predominantly membrane associated after a pulse as brief as 2.5 min, suggesting either that P68 may be synthesized on membrane-bound polyribosomes or that P68 is made on free polyribosomes but is transported extremely rapidly to the membrane. No proteolytic cleavage of P68 was observed.

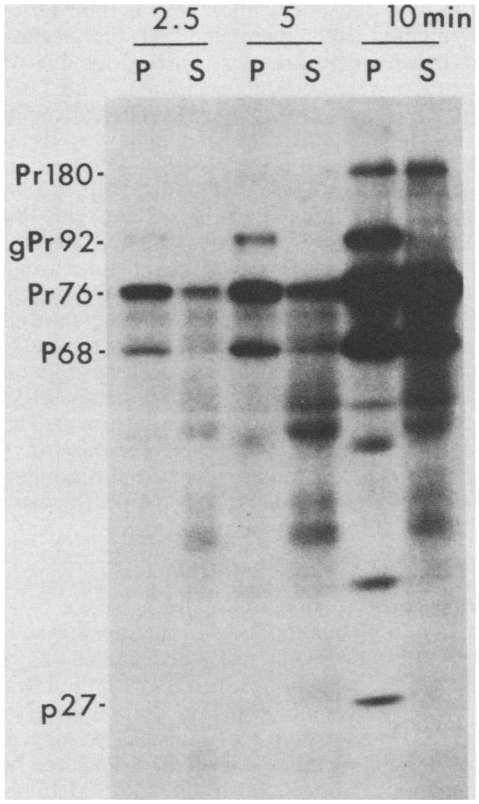

FIG. 2. Subcellular distribution of pulse-labeled P68. UR2-transformed cells were pulse-labeled with [^{35}S]methionine for the indicated amounts of time, Dounce homogenized, and separated into crude membrane (P) and cytosolic (S) fractions by differential centrifugation. Fractions were extracted into RIPA buffer and immunoprecipitated with TBR serum.

The transforming protein p60^{src} of RSV transiently associates with two cellular proteins, p50 and P90, to form a complex thought to be involved in the transport of p60^{src} to the plasma membrane (7, 8, 14). A minor population of the transforming proteins of Fujinami (P140$^{gag\text{-}fps}$) and Yamaguchi Y73 (P90$^{gag\text{-}yes}$) sarcoma viruses is also found associated with cellular proteins p50 and p90 (31), suggesting a common role for the interaction of p50 and p90 with viral transforming proteins encoding tyrosine kinases. We have examined whether P68, which shares extensive amino acid sequence homology with *src*, *fps*, and *yes* in the kinase domain, associates with p50 and p90. No specific coprecipitation of p50 and p90 with P68 could be detected in immunoprecipitates from whole cell lysates from UR2-transformed cells (Fig. 1). A lysate from [^{3}H]leucine-labeled UR2-transformed cells was sedimented through a glycerol gradient, and gradient fractions were immunoprecipitated (Fig. 3). A single peak corresponding to monomer P68 was observed; even upon long exposure, no fast-sedimenting P68 complexed to p50 and p90 could be detected. Sedimentation of P68 as a monomer species was also observed upon glycerol gradient analysis of a lysate from UR2-transformed cells pulse-labeled with [^{3}H]leucine for 15 min (data not shown), labeling conditions in which newly synthesized p60^{src} in RSV-transformed cells was readily detected in a fast-sedimenting form (data not shown). Upon chase (various periods up to 4 h), pulse-labeled P68 continued to behave as a monomer species upon gradient analysis (data not shown). At no time during its lifetime was P68 detected in a complex with p50 and p90.

Biological characterization of UR2 *ts* mutants. To analyze the process of transformation by UR2, we isolated and characterized three *ts* mutants of UR2. Virus obtained by treatment of UR2-infected cultures with 5-azacytidine was used for colony formation, and individual colonies were examined for the temperature sensitivity of their morphological alteration of infected cells. We screened about 400 colonies induced by UR2 treated with 5-azacytidine. Twelve clones appeared to be *ts* in morphological changes. Three viruses, called tsNY251, tsNY252, and tsNY253, were studied. Infected cells derived from colonies of tsNY251, tsNY252, and tsNY253 were morphologically different at 37 and 42°C; cultures displayed the characteristic elongated UR2-transformed morphology at 37°C but were flat at 42°C (data not shown). The morphological state could be reversed repeatedly by temperature shift (data not shown).

Virus production by cells transformed with tsNY251 and tsNY252 was identical at 37 and 42°C and comparable with that by wt-UR2-infected cells (data not shown). However, the production of transforming virus from tsNY253 at 42°C was reduced to 10% of the virus production at 37°C (data not shown). The reduction of UR2 virus titer in this case was not due to a reduction in helper virus production; UR2AV was not *ts* in replication (data not shown).

The abilities of these mutants to produce colonies in soft agar at the permissive and nonpermissive temperatures are shown in Table 3. The efficiency of colony formation was greatly reduced at the nonpermissive temperature.

Membrane localization of *ts* mutant P68. tsNY251-, tsNY252-, and tsNY253-infected cells produced immunoprecipitable P68 at both temperatures (data not shown), but there was a 10 to 35% decrease in the total

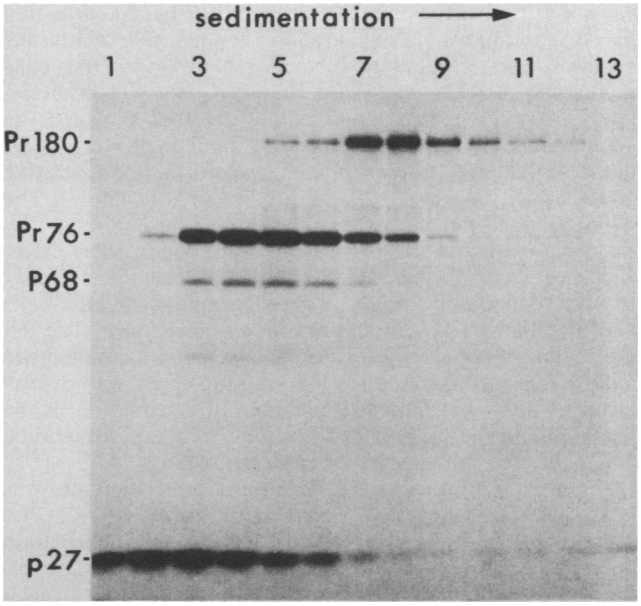

FIG. 3. Glycerol gradient sedimentation analysis of a lysate from UR2-transformed cells. A RIPA lysate from UR2-transformed cells labeled for 4 h with [^{3}H]leucine was layered onto a 10 to 30% glycerol gradient in RIPA buffer and centrifuged for 17 h at 44,000 rpm at 4°C in an SW50.1 rotor. Gradient fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right.

TABLE 3. Colony formation in soft agar by UR2 *ts* mutants

Virus	Dilution	No. of colonies at (°C):	
		37	42
wt	10^{-1}	TMTC[a], TMTC	TMTC, TMTC
	10^{-2}	1,100, 850	910, 550
tsNY251	10^{-1}	TMTC, TMTC	2, 8
	10^{-2}	595, 420	0, 0
	10^{-3}	91, 75	0, 0
tsNY252	10^{-1}	TMTC, TMTC	6, 0
	10^{-2}	450, 380	0, 0
	10^{-3}	121, 60	0, 0
tsNY253	10^{-1}	TMTC, TMTC	3, 0
	10^{-2}	1,200, 880	0, 0
	10^{-3}	200, 150	0, 0

[a] TMTC, Too many to count.

amount of P68 (relative to viral structural proteins Pr76 and p27) at the nonpermissive temperature. The decrease in P68 was somewhat variable, but the extent of decrease was not likely to be significant enough to account for the loss of the transformed phenotype at the nonpermissive temperature.

For a number of *ts* and nonconditional nontransforming *src* mutants of RSV, the loss of transforming activity is associated with an alteration in the subcellular localization of the transforming protein from membrane associated to soluble (7, 14, 16, 22, 37). To determine whether a change in membrane association accounted for the behavior of the *ts* UR2 mutants, UR2-infected cells maintained at the permissive or nonpermissive temperature were labeled with [^{3}H]leucine for 4 h and fractionated (Fig. 4). The P68 protein in wt and *ts* mutant NY251-, NY252-, and NY253-infected cells was membrane associated at both temperatures. The membrane association at the nonpermissive temperature was not salt sensitive (data not shown). Further fractionation on a discontinuous sucrose gradient of the crude membrane fraction from [^{3}H]leucine-labeled tsNY252-infected cells maintained at 42°C suggested that P68 was still predominantly plasma membrane associated (data not shown).

Kinase activity of *ts* mutants. In a number of *src* and *fps* mutants that are *ts* for cellular transformation, tyrosine kinase activity is *ts* (10, 22, 23, 30, 35, 41, 44, 45, 47). The P68-associated kinase activity from *ts*-mutant-infected cultures maintained at the permissive and nonpermissive temperatures was measured (Table 4). P68 protein kinase activity was significantly reduced when *ts*-mutant-infected cells were incubated at the nonpermissive temperature. The decrease in phosphorylation of the exogenous substrate enolase and in P68 autophosphorylation suggests an overall decrease in enzyme activity not merely a reduction in the phosphoacceptor capacity of P68 due to a conformational change at the nonpermissive temperature.

The in vitro protein kinase activity of the *ts* mutants was more sensitive to heat inactivation than that of wt UR2 (Fig. 5). The kinetics of heat inactivation of P68 phosphorylation were similar to those of enolase phosphorylation. The different *ts* mutants had reproducibly different kinetics of heat inactivation.

In many *src* and *fps* mutants *ts* for cellular transformation, tyrosine phosphorylation of the transforming protein is greatly reduced at the nonpermissive temperature (10, 23, 30, 35, 47). P68 is a phosphoprotein in vivo, phosphorylated both on serine and tyrosine residues (20). To examine the nature of in vivo phosphorylation of *ts* mutant P68, wt- and *ts*-mutant-infected cells maintained at the permissive or nonpermissive temperature were labeled for 4 h with $^{32}P_i$, and cell extracts were immunoprecipitated with TBR serum. Immunoprecipitates were split and analyzed on duplicate SDS-polyacrylamide gels, one of which was treated with alkali (12), to enrich for phosphotyrosine-containing phosphoproteins (data not shown). Based upon comparison of the intensities of the bands of specific phosphoserine-only-containing viral structural proteins in autoradiographs of different exposure times of the treated and untreated gels, it was determined that alkali treatment removed greater than 90% of the phosphoserine. While wt UR2 showed an increase in the intensity of the alkali-resistant P68 band, all three *ts* mutants showed a significant decrease (greater than fivefold) in the intensity of the alkali-resistant P68 band at the nonpermissive temperature (data not shown). The results suggested that there was a marked decrease in tyrosine phosphorylation of the mutant P68s at the nonpermissive temperature, consistent with the decreased kinase activity observed in vitro.

DISCUSSION

Membrane association of P68. The presence of a hydrophobic domain long enough to span the membrane at the N terminus of the *ros*-specific sequence in P68 (33) and the amino acid sequence homology of P68 with the intracellular tyrosine kinase domains of the epidermal growth factor receptor (17, 51) and insulin receptor (18, 50) suggested that P68 is an integral membrane protein associated with the plasma membrane. Subcellular fractionation data presented here suggest that P68 is an integral plasma membrane-associated protein. Our data did not address questions concerning P68 expression on the cell surface and its orientation within the membrane. However, preliminary cell surface iodination experiments and indirect immunofluorescence localization studies with p19-specific antiserum on fixed, nonpermeabilized, nonproducer UR2-transformed cells suggest that P68 does span the plasma membrane with its p19 sequences exposed extracellularly (S.-M. Jong and L.-H. Wang, personal communication).

P68 apparently is not modified with covalently bound fatty acid as are the plasma membrane-associated transforming proteins of RSV, Abelson murine leukemia virus, and Harvey sarcoma virus (42, 46). N-terminal myristylation of P68 was not likely, since P68 lacks an N-terminal glycine acceptor residue (33) that appears to be required for N-myristylation (16, 36, 37, 42). Evidently P68 is not a glycoprotein as are the plasma membrane-associated transforming proteins of avian erythroblastosis virus (24, 26, 38) and the McDonough strain of feline sarcoma virus (1, 2, 40). P68 contains only one potential site for addition of N-linked oligosaccharides: at amino acid 444, which is located within the conserved tyrosine kinase domain (33). The glycosylated transforming proteins of avian erythroblastosis virus (24, 26, 38) and the McDonough strain of feline sarcoma virus (1, 2, 40) and the glycosylated transmembrane receptors for epidermal growth factor (17, 26, 51) and insulin (18, 50) are glycosylated within their extracellular domains but not within the conserved tyrosine kinase domains.

Original stocks of UR2 (UR2AV) apparently contain electrophoretically variant forms of P68 (this work; 20, 32). Polymorphism in electrophoretic mobility of transforming proteins has been observed in RSV (5) and Fujinami sarcoma virus (19, 21, 23, 30, 35). For RSV, sequence analysis of different strains suggests that the polymorphism is due to a

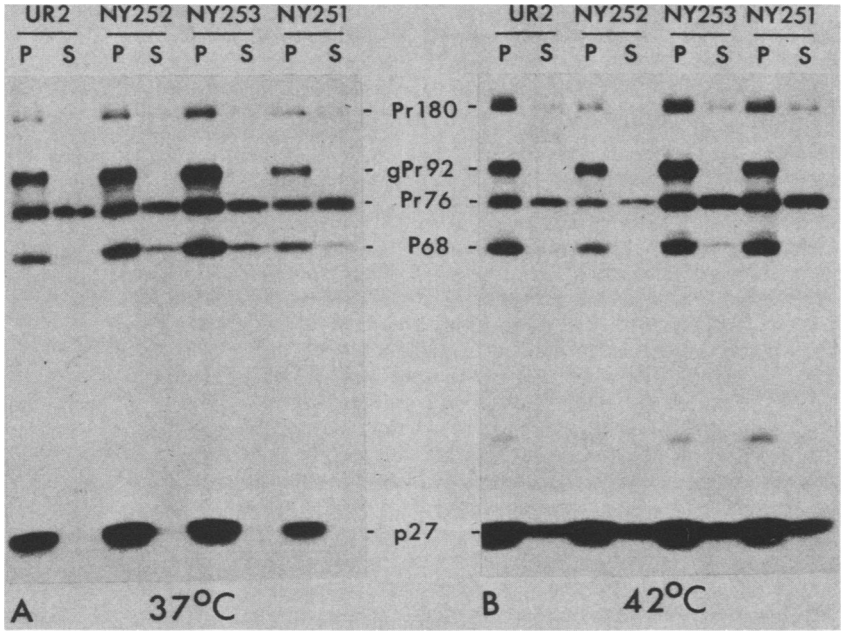

FIG. 4. Membrane association of P68 in *ts* mutant virus-infected cells. wt or *ts* mutant virus-infected cells maintained at 37 (A) or 42°C (B) were labeled with [^{3}H]leucine for 4 h, Dounce homogenized, and separated into crude membrane (P) and cytosolic (S) fractions by differential centrifugation. Fractions were extracted into RIPA buffer and immunoprecipitated with TBR serum.

number of amino acid substitutions rather than deletions or insertions (43, 48, 49).

Transport of P68 to the membrane. Our data suggest that P68 becomes membrane associated very rapidly. P68 is translated from a 24S genomic RNA whose 5′ noncoding sequences resemble those of avian retroviral *gag*-encoding RNA (33, 52). Fractionation of polyribosomes suggests that the majority of Pr76^{gag} protein is synthesized on free polyribosomes (29, 39), suggesting that it is likely that P68 is also synthesized on free polyribosomes. However, there are a number of sequence changes within the UR2 5′ noncoding sequence (33) which could possibly direct the translation of P68 on membrane-bound polyribosomes.

In vitro translation (20) and sequence analysis (33) indicate that P68 contains no cleavable signal sequence that could account for its rapid membrane association. The hydrophobic sequence at the N terminus of the *ros*-specific sequence may serve as an internal membrane insertion anchor signal (6). This mechanism has a precedent; it has been suggested that an N-terminal hydrophobic sequence in the influenza virus M2 protein, whose sequence also lacks a canonical signal sequence, serves as an anchor for this integral membrane protein that is expressed on the surface of influenza virus-infected cells (28).

TABLE 4. Protein kinase activity of *ts* mutants

UR2 strain used	42°C/37°C ratio of ^{32}P (cpm) incorporated into[a]:	
	P68	Enolase
wt	1.06	1.01
tsNY251	0.13	0.17
tsNY252	0.15	0.18
tsNY253	0.13	0.18

[a] Infected cells maintained at 37 or 42°C were extracted into RIPA buffer lacking SDS. Portions containing equivalent amounts of total cell protein from extracts of infected cells maintained at 37 or 42°C were immunoprecipitated with TBR serum and analyzed for in vitro protein kinase activity. The autophosphorylation of P68 in immune complexes was unaffected by the presence of the exogenous substrate enolase. Values shown represent the average of at least two independent determinations.

No evidence could be found for P68 association with two cellular proteins, p50 and p90, that associate with the transforming proteins encoded by *src*, *fps*, and *yes* (8, 31) and are thought to be involved in the transport of p60^{src} to the plasma membrane (7, 14). It has been suggested that the association of p50 and p90 with tyrosine kinases is mediated through C-terminal sequences within the conserved kinase domain (7, 31). P68 contains unique amino acid changes and insertions within the kinase domain (33) that could preclude association with p50 and p90.

***ts* UR2 mutants.** We have isolated and characterized three mutants of UR2 that are *ts* for cellular transformation by the criteria of morphological alteration and anchorage-independent growth. As observed for many *src* and *fps ts* mutants (10, 23, 30, 35, 41, 44, 45, 47), the tyrosine phosphorylation of the transforming protein and the tyrosine kinase activity are *ts*. However, membrane association of P68 was not *ts*, unlike the change in localization of the p60^{src} that is associated with the loss of transforming capacity observed in a number of *ts* and nonconditional transformation-defective *src* mutants (7, 14, 16, 22, 37). Considering the relative target sizes of the 5′ hydrophobic domain that appears to mediate P68 membrane association and the kinase domain and considering the selection for temperature sensitivity of transformation applied during isolation of the *ts* mutants, it is not surprising that the mutants are *ts* for kinase activity but are not *ts* for membrane association of P68. These mutants provide genetic evidence that P68 kinase activity is crucial for cellular transformation by UR2.

Although elevation of total cellular phosphotyrosine appears to be essential for transformation mediated by *src* or *fps* (13, 23, 45, 47), it has been reported that UR2-transformed cells show no significant elevation of total cellular phosphotyrosine (13). UR2-transformed cells maintain a higher level of cytoskeletal organization than RSV-

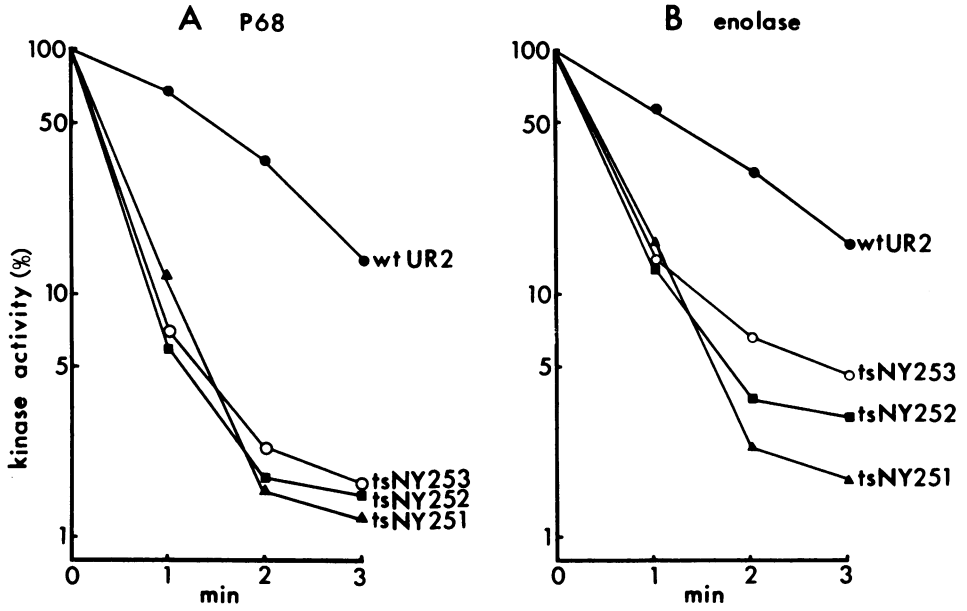

FIG. 5. Heat inactivation of kinase activity. Infected cells maintained at 37°C were lysed in RIPA buffer without SDS. Portions containing equal amounts of total cell protein were incubated at 41°C for 1, 2, or 3 min. Heat inactivation was stopped by the addition of cold buffer, and samples were immunoprecipitated with TBR serum and subjected to kinase assay in the presence of the exogenous substrate enolase. (The amount of total immunoprecipitable [^{3}H]leucine-labeled P68 was not significantly reduced by heat inactivation.) The amount of ^{32}P radioactivity transferred to P68 (A) or enolase (B) was determined. Results are expressed as the percentage of residual kinase activity relative to that in an unheated sample.

transformed cells (3, 34) and express more cell surface fibronectin than normal cells (34), suggesting that P68 has a spectrum of substrates distinct from those of other transforming proteins. A limited substrate specificity for P68 is also suggested by the observation that tyrosine phosphorylation on vinculin, a substrate common to several viral tyrosine kinases (see reference 13 for a review), is not elevated in UR2-transformed cells (3). Further studies utilizing the UR2 *ts* mutants should elucidate the nature of these differences.

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