Analysis of p60^{v-src} Mutants Carrying Lesions Involved in Temperature Sensitivity

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Analysis of the *src* genes of three temperature-sensitive (*ts*) mutants of Rous sarcoma virus (*ts*NY68, *ts*NY72-4, and PA104) showed that each has two C-terminal mutations in the kinase domain required for temperature sensitivity, as assayed by morphological alteration and anchorage-independent growth. In all three mutants, one of the mutations is a valine-to-methionine change at position 461. To assess the contribution of each mutation to the biochemical properties of the *src* protein, we analyzed the kinase activity and the interaction with cellular proteins p50 and p90 of recombinant *src* gene products in which only one mutation was combined with wild-type *src* sequences. Chimeric *src* protein containing only the Met-461 mutation was indistinguishable from the wild type by all criteria examined, while the effect of the second C-terminal mutation alone varied with the defectiveness of the parental *ts* mutant. The second mutation alone, while not sufficient to cause *ts* transformation, altered p60^{src} complex formation with cellular proteins p50 and p90 and altered the *in vitro* thermolability of *src* kinase activity. The results indicate that these biochemical properties of p60^{src} are more sensitive to mutation than others, such as *in vivo* kinase activity, which require more profound structural alterations.

Temperature-sensitive (*ts*) transformation mutants of Rous sarcoma virus (RSV) have been useful in the identification and delineation of the functions of the transforming gene product p60^{src} (for reviews, see references 1 and 24). The dissociation of transformation parameters exhibited by *ts* and nonconditional mutants has indicated that p60^{src} has multiple functional domains and targets (1, 7, 24). Molecular cloning and sequencing of the *src* genes of *ts* mutants has provided precise definitions of genetic lesions conditionally affecting p60^{src} function (11, 26, 27).

The *src* genes of three independently isolated and phenotypically different (4, 20, 21, 26, 27, 30) *ts* mutants (*ts*NY68, *ts*NY72-4, and PA104) derived from Schmidt-Ruppin subgroup A RSV (SR-RSV-A) recently have been sequenced, and the contribution of individual mutations to the *ts* phenotype has been assessed by *in vitro* recombination with wild-type (wt) *src* sequences (26, 27). In all three mutants, there are two lesions conferring temperature sensitivity located in the C-terminal kinase domain of p60^{src} (26, 27). In all three mutants, one of the two mutations is a valine-to-methionine change at position 461. The phenotype of cells infected with virus carrying a chimeric *src* gene containing only the Met-461 mutation was indistinguishable from that of wt infected cells, while the effect of the second C-terminal mutation alone varied in a temperature-independent manner with the defectiveness of the parental *ts* mutant (26, 27).

The exact mechanism of temperature sensitivity is unknown. However, the three aforementioned *ts* mutants have defects in both kinase activity and p60^{src} membrane association, two functions of p60^{src} necessary for transformation (1, 2, 8, 9, 14, 18, 19, 22, 32). The defect in membrane association appears to be due to aberrant binding to two cellular proteins, p50 and p90, thought to be involved in the transport of p60^{src} to the membrane (2, 3, 8). To assess the

contribution of each mutation to these properties of p60^{src} and to study the nature of temperature sensitivity, we examined complex formation with p50 and p90 and the kinase activity of the parental and recombinant wt-*ts* *src* proteins. We found that the Met-461 mutation alone has no effect on the properties examined but that the second C-terminal mutation alone affects p50-p90 complex binding and the *in vitro* thermolability of kinase activity. Both C-terminal mutations are required for full temperature sensitivity *in vivo*.

MATERIALS AND METHODS

Cell cultures and viruses. Cultures of secondary chicken embryo fibroblasts (CEF) were maintained and infected as previously described (16). Cloned *src* genes were expressed by using the two-plasmid vector system previously described (10) to generate infectious virus. Culture media harvested 7 to 12 days posttransfection were used to infect CEF.

Plasmid constructions. Standard recombinant DNA techniques were used to construct the *src*-containing plasmids used in this study (Fig. 1). All *ts* and recombinant wt-*ts* *src* genes were cloned into the homologous site of pSR-XD2, which contains the wt v-*src* gene of SR-RSV-A (10, 26). pXD68, pXD72, and pXD104 contain the *ts* *src* genes of *ts*NY68, *ts*NY72-4, and PA104, respectively (19, 26). The construction of recombinants pXD372 (containing only the two C-terminal mutations of *ts*NY72-4), pXDM72 (containing only the Met-461 mutation), pXDS72 (containing only the Ser-503 mutation), and pXDP104 (containing only the Pro-325 mutation), has been previously described (26). pXDD68, containing only the deletion of positions 352 to 354 of *ts*NY68 was made by three-part ligation of the 3.0-kilobase *Cla*I-*Bgl*II and 4.9-kilobase *Sac*I-*Cla*I fragments of pXD68 with the 1.3-kilobase *Bgl*II-*Sac*I fragment of pSR-XD2.

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FIG. 1. Recombinant *src*-containing plasmids and the viruses derived from them. Recombinant *src* genes, the corresponding replication-competent viruses derived from them, and the phenotypes of the viruses are shown. The structure of $p60^{src}$ is indicated at the top of the figure. The wavy line denotes myristic acid linked to glycine 2. ATP represents the ATP-binding site at lysine 295. Y represents the major site of tyrosine phosphorylation at tyrosine 416. Below the $p60^{src}$ structure are the structures of chimeric *src* genes. $\square$, Sequences derived from *ts*NY68; $\square$, sequences derived from *ts*NY72-4; $\square$, sequences derived from PA104; $\square$, sequences derived from wt SR-RSV-A (SRA). Open arrowheads indicate mutations relative to wt SR-RSV-A. Cleavage sites for restriction enzymes used to construct *ts* and hybrid wt-*ts src* genes into the homologous site of pSR-XD2 are indicated. NYS72 is slightly *ts* (26). NYPA104 is both *ts* and quite transformation defective (d) (4, 20, 26). NYP104 is similar to NYPA104 but is much less *ts* (26).

Labeling conditions, immunoprecipitation, and kinase assay. Cultures were labeled and lysates were prepared as previously described (12, 13). For kinase assays, RIPA lacking both sodium dodecyl sulfate (SDS) and sodium deoxycholate was used (for preparation of lysates and for washing immunoprecipitates) to prevent denaturation of kinase activity (31). Lysates were immunoprecipitated with saturating amounts of antiserum as previously described (13). For immunoprecipitation of $p60^{src}$, serum from a rabbit bearing a tumor induced by Schmidt-Ruppin subgroup D RSV (TBR serum) or monoclonal antibody (MAb) 327 (kindly provided by J. Brugge [25]) was used. The mutant *src* proteins were immunoprecipitated equally efficiently by both TBR serum and MAb 327, although the TBR serum used did not cross-react with the *c-src* protein. For immunoprecipitation of the 34K *src* substrate, rabbit antiserum to purified chicken 34K protein (kindly provided by M. Greenberg [15]) was used. Immunoprecipitates were processed and analyzed on 10% SDS-polyacrylamide gels as previously described (13). Kinase assays were done as previously described (13), except that the kinase buffer contained 10 mM Tris hydrochloride (pH 7.4) and 5 mM $MgCl_2$ and reactions proceeded for 10 min. V8 protease analysis of labeled $p60^{src}$ was done as previously described (12).

Cell fractionation and gradient analysis. Cells labeled for 4 h with $[^3H]$ leucine were fractionated by differential centrifugation into particulate and cytosolic fractions as previously described (9, 12). Fractionation of cell extracts on glycerol gradients was done as previously described (2, 12).

RESULTS

Properties of *ts* and recombinant wt-*ts src* genes. A comparison of the DNA sequences of the molecularly cloned *src* genes of wt SR-RSV-A, *ts*NY68, *ts*NY72-4, and PA104 (26, 27) and in vitro recombination studies (Fig. 1) showed that the three *ts* mutants each contain two amino acid substitutions in the C-terminal kinase domain that confer temperature sensitivity (26, 27). Both mutations are required for full temperature sensitivity, as assayed by morphological transformation and anchorage-independent growth. One of the mutations is an identical valine-to-methionine change at position 461. Each of the mutants contains a second C-terminal mutation required for the *ts* phenotype: *ts*NY68 contains a deletion of the sequence Gly-Glu-Met at positions 352 to 354, *ts*NY72-4 contains a proline-to-serine substitution at position 503, and PA104 contains a leucine-to-proline substitution at position 325 (26, 27). NYM72, containing only the Met-461 mutation, is phenotypically indistinguishable from the wt. The second mutation alone affects transformation in a temperature-independent manner in which morphological alteration varies with the defectiveness of the parental *ts* mutant (26, 27). NYD68, containing only the deletion of positions 352 to 354, is indistinguishable from the wt. NYS72, containing only the Ser-503 mutation, confers a slightly flatter morphology than the wt. NYP104, containing only the Pro-325 mutation, confers an extremely subtle morphological alteration dependent upon culture conditions (20, 26). These mutants, unlike the parental *ts* mutants,

FIG. 2. $p60^{src}$ production in CEF infected with RSV variants. CEF infected with RSV variants were labeled for 4 h at 37°C with [3 H]leucine and lysed in RIPA buffer. Portions of lysates containing equal amounts of total cell protein were immunoprecipitated with MAb 327. SRA, SR-RSV-A.

which also encode the Met-461 mutation, are not significantly temperature sensitive for anchorage-independent growth (26).

We examined the production and stability of $p60^{src}$ in cells infected with the wt, *ts* variants, and hybrid wt-*ts* RSV variants. Infected CEF were labeled for 4 h at 37°C with [3 H]leucine, and $p60$ s were immunoprecipitated with MAb 327 (Fig. 2). Similar levels of $p60^{src}$ production were observed among the variant viruses. The $p60^{src}$ levels were similar at 41°C (data not shown). The half-lives of the *ts* and hybrid wt-*ts* mutants at both 37 and 41°C were examined by pulse-chase analysis (data not shown). The stabilities of the

mutant $p60$ s were not significantly different from that of the wt $p60$ and were not significantly affected by growth temperature.

Complex formation and membrane association. Pulse-chase and cell fractionation studies indicated that newly synthesized $p60^{src}$ transiently associates with two cellular proteins, p50 and p90, and is rapidly transported to the plasma membrane, where $p60^{src}$ is released from the soluble complex (2, 8). In many *ts* mutants, the stability of the $p60^{src}$ -p50-p90 complex is increased, and binding to the membrane is impaired at the nonpermissive temperature (2, 8, 14). More p50 and p90 coprecipitated with the *src* proteins of the parental *ts* mutants NY68, NY72, and NYPA104 and the hybrid mutants NYD68, NYS72, and NYP104 than with the *src* protein of the wt or mutant NYM72 at 37°C (Fig. 2), suggesting that the second strain-specific C-terminal mutation results in increased $p60^{src}$ -p50-p90 complex stability. The increase in coprecipitating p50 and p90 relative to that seen in wt- or NYM72-infected cells was also seen with longer labeling periods and with labeling at 41°C (data not shown).

Figures 3B and C show that at 37°C virtually all of the NY72 $p60^{src}$ was found in a fast-sedimenting form, complexed with p50 and p90, while most of the NYM72 $p60^{src}$ sedimented as a monomer, like wt $p60^{src}$ (data not shown). In glycerol gradient sedimentation analysis, the *src* proteins of NY68, NYD68, NY72, NYS72, NYPA104, and NYP104 were found predominantly in the fast-sedimenting complex, regardless of growth temperature, labeling period, or chase period (data not shown), while the sedimentation behavior of NYM72 $p60^{src}$ was similar to that of wt $p60^{src}$. These results suggest that binding to the p50-p90 complex is irreversible for *src* mutants carrying the second C-terminal mutation.

We examined the membrane association of the variant $p60$ s by subcellular fractionation. Infected cells were labeled with [3 H]leucine, homogenized, and separated into crude membrane and soluble fractions by differential centrifugation, and fractions were immunoprecipitated with TBR serum (Fig. 3A and data not shown). wt and mutant NYM72 $p60$ s behaved similarly: they were predominantly membrane

FIG. 3. Analysis of fractionation pattern and glycerol gradient sedimentation behavior of mutant *src* proteins. CEF infected with RSV variants were labeled for 4 h at 37°C with [3 H]leucine. (A) Cells were homogenized and separated into crude membrane (P) and soluble (S) fractions by differential centrifugation. Fractions were solubilized in RIPA buffer and immunoprecipitated with TBR serum. (B and C) RIPA buffer lysates of infected cells were analyzed by sedimentation through 10 to 30% glycerol gradients. Fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right. Fraction numbers are indicated at the top. SRA, SR-RSV-A.

FIG. 4. Glycerol gradient sedimentation analysis of separated membrane and soluble fractions. NY72-infected cells maintained at 37°C were labeled and fractionated as described in the legend to Fig. 3. Crude membrane (P100) and soluble (S100) fractions were solubilized in RIPA buffer and sedimented through 10 to 30% glycerol gradients. Fractions were immunoprecipitated with TBR serum. Sedimentation was from left to right. Fraction numbers are indicated.

associated regardless of growth temperature. NY68 and NY72 showed a decrease in membrane-associated $p60^{src}$ at the nonpermissive temperature (data not shown; 2, 8, 14). NYPA104 showed a temperature-independent decrease in membrane-associated $p60^{src}$ (19). Hybrid proteins containing the second C-terminal mutations had temperature-independent fractionation patterns similar to the pattern of the parental *ts* mutants at the permissive temperature; e.g., NYS72 and NYD68 were membrane associated, while NYP104 showed a decrease in membrane-associated $p60^{src}$ (data not shown).

The glycerol gradient sedimentation behavior of separated crude membrane and soluble fractions was examined. Figure 4 shows an analysis of fractions from NY72-infected cells. $p60^{src}$ from both fractions was in a fast-sedimenting form complexed to p50 and p90. Similar behavior was observed for the *src* proteins of NYS72, NY68, NYD68, NYPA104, and NYP104 (data not shown). For both wt and NYM72, only the soluble $p60^{src}$ was in the complex, while the membrane-associated $p60^{src}$ sedimented as a monomer (data not shown). For NY68, NYD68, NY72, NYS72, NYPA104, and NYP104, the $p60^{src}$ in the crude membrane pellet remained complexed to p50 and p90 through further fractionation by flotation in discontinuous sucrose gradients (data not shown). These results suggest that the second C-terminal mutation results in stable complex formation that is not dissociated at the plasma membrane.

Myristylation is necessary for $p60^{src}$ membrane association (9, 12). No differences in labeling with [3 H]myristic acid of the *src* proteins of the wt, *ts* variants, or hybrid wt-*ts* variants were observed (data not shown), as expected, since

the extreme N-terminal sequence, containing the myristylation signal for $p60^{src}$ (9, 12, 29), is identical in all the variants. Differences in membrane association are apparently not due to differences in myristylation of the mutant proteins.

In vitro kinase activity of the mutants. Table 1 shows the results of in vitro kinase assays performed on mutant *src* proteins immunoprecipitated with TBR serum from infected cell lysates. NY68 and NY72 showed a decrease in kinase activity at 41°C as compared with 37°C. NYPA104 and NYP104 showed greatly reduced levels of kinase activity regardless of temperature. NYD68, NYM72, and NYS72 showed no temperature sensitivity in kinase activity.

Because the assay with tightly bound TBR immunoglobulin G (IgG) as a substrate is somewhat artificial, we also measured in vitro autophosphorylation and phosphorylation of the exogenous substrate enolase in immune complexes formed with MAb 327 (data not shown). Results were similar to those from the TBR IgG kinase assay.

We found no intrinsic defect in kinase activity in p50-p90 complex-bound $p60^{src}$. A TBR immune complex kinase assay of fractions immunoprecipitated from glycerol gradients showed levels of kinase activity in fast-sedimenting $p60^{src}$ consistent with the kinase activity seen in whole-cell lysates from NY68-, NYD68-, NY72-, NYS72-, NYPA104-, or NYP104-infected cells (data not shown). For the kinase assay, immune complexes were washed in RIPA buffer without SDS or sodium deoxycholate, in contrast to the use of complete RIPA by Brugge et al. (2, 3) and Courtneidge and Bishop (8), who failed to detect kinase activity in the complex.

We compared the in vitro thermolability of the kinase activity of the variants (Fig. 5). The parental *ts* mutants and hybrids containing the second C-terminal mutation alone were more labile to heat inactivation than the wt or NYM72, containing only the Met-461 mutation. The increased thermolability correlated with an increased binding to p50 and p90, suggesting that complex-bound $p60^{src}$ is more susceptible to denaturation.

In vivo kinase activity of the variants. We examined the alkali-resistant phosphorylation in infected cells of the 34K protein, a membrane-associated cellular protein that is a

TABLE 1. Tyrosine kinase activities of mutant *src* proteins

Virus	TBR IgG phosphorylation (fraction of SR-RSV-A) at ^a :		Alkali-resistant 34K protein (fraction of SR-RSV-A) at ^b :	
	37°C	41°C	37°C	41°C
SR-RSV-A	1.00	1.00	1.0	1.0
NY72	1.00	0.47	0.5	0
NYM72	1.00	1.00	1.0	1.0
NYS72	1.00	1.00	0.5	0.4
NY68	1.00	0.45	0.5	0.1
NYD68	1.00	1.00	0.5	0.3
NYPA104	0.01	0.01	0.2	0
NYP104	0.02	0.02	0.2	0.2

^a Infected cells maintained at 37 or 41°C were lysed in RIPA buffer lacking both SDS and sodium deoxycholate. Portions of cell lysates containing equal amounts of total cell protein were immunoprecipitated with TBR serum and subjected to a kinase assay. Values represent the fraction of TBR IgG phosphorylation relative to that in SR-RSV-A-infected cells.

^b Infected cells maintained at 37 or 41°C were labeled for 6 h with 32 P_i. Portions of cell lysates containing equal amounts of total cell protein were immunoprecipitated with rabbit anti-chicken 34K protein serum (15). Immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels, which were alkali treated to enrich for phosphotyrosine-containing proteins (7). 32 P-labeled 34K bands were excised, and radioactivity was quantitated. Values represent the fraction of alkali-resistant 34K protein relative to that in SR-RSV-A-infected cells.

FIG. 5. Heat inactivation of kinase activity. (A) Infected cells maintained at 37°C were lysed in RIPA buffer without SDS or sodium deoxycholate. 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 a kinase assay. (B to D) The amount of ^{32}P radioactivity transferred to TBR IgG was determined. Results are expressed as the percentage of residual kinase activity relative to that in an unheated sample for each mutant. SRA, SR-RSV-A.

major substrate of tyrosine-specific protein kinases (7, 15). The 34K protein was immunoprecipitated from $^{32}\text{P}_i$ -labeled cell lysates, and after gel electrophoresis, the gels were treated with alkali to enrich for phosphotyrosine-containing phosphoproteins (7). The 34K protein-containing region of the gel was excised, and radioactivity was quantitated. The results are shown in Table 1. Alkali-resistant phosphorylation of the 34K protein was temperature sensitive in the parental *ts* mutants but not significantly temperature sensitive in the hybrid *wt-ts* mutants. NYPA104 and NYP104 showed very low levels of 34K protein alkali-resistant phosphorylation, consistent with their low *in vitro* kinase activities. The decrease in 34K protein alkali-resistant phosphorylation correlated with the phenotype of mutant-infected cells.

We also examined phosphorylation of the mutants p60^{src} by V8 partial proteolytic cleavage analysis of ^{32}P -labeled p60^{src} (Fig. 6). V8 protease cleavage of p60^{src} yields N-terminal fragments V1, V3, and V4 containing the major phosphoserine site and C-terminal fragment V2 containing the major phosphotyrosine site (6, 17). For many *ts* mutants, V2 phosphorylation correlates with transforming activity (2, 8, 34). The *wt* and NYM72 showed comparable phosphorylation patterns at both temperatures. For NY68 and NY72, a

significant decrease in V2 phosphorylation relative to V1 phosphorylation was seen at the nonpermissive temperature. NYD68 and NYS72 showed a slight decrease in V2 phosphorylation at 41°C. NYPA104 and NYP104 were severely underphosphorylated on V2 and somewhat underphosphorylated on V1 at both temperatures. The phosphorylation of V2 was alkali resistant for all the mutants (data not shown).

DISCUSSION

To dissect the nature of temperature sensitivity, to assess the contribution of individual mutations to the defects in kinase activity and membrane association observed in three cloned and sequenced *ts src* mutants, and to correlate biochemical defects with transforming activity, we examined the biochemical properties of mutants containing recombinant *wt-ts src* genes. Table 2 summarizes the data.

tsNY68, *tsNY72-4*, and PA104 each contain a change from valine to methionine at position 461 in addition to a second strain-specific C-terminal mutation (26, 27). The Met-461 mutation alone had no effect on any of the parameters examined. For all three mutants, the second C-terminal mutation alone affected complex formation and the *in vitro* thermolability of kinase activity. Both mutations are re-

TABLE 2. *p60^{src}* properties in mutant-infected cells^a

Virus	Transformation	Kinase activity				Stable complex formation ^b
		In vitro		In vivo		
		TBR IgG phosphorylation	Thermolability	Autophosphorylation	34K Phosphorylation	
NY72	<i>ts</i>	<i>ts</i>	<i>ts</i>	<i>ts</i>	Low, <i>ts</i>	+
NYM72	wt	wt	wt	wt	wt	—
NYS72	wt ^c	wt	<i>ts</i>	wt ^d	Low	+
NY68	<i>ts</i>	<i>ts</i>	<i>ts</i>	<i>ts</i>	Low, <i>ts</i>	+
NYD68	wt	wt	<i>ts</i>	wt ^d	Low	+
NYPA104	<i>ts</i> , d ^e	Low	<i>ts</i>	Low	Low, <i>ts</i>	+
NYP104	d ^f	Low	<i>ts</i>	Low	Low	+

^a wt and *ts* indicate the absence and presence of temperature sensitivity, respectively.

^b +, Stable complex formation; —, transient complex formation.

^c NYS72 is slightly *ts* for morphological transformation (26).

^d NYS72 and NYD68 are slightly *ts* for autophosphorylation.

^e NYPA104 is defective (d) for morphological transformation at 37°C (19, 26).

^f NYP104 is similar to NYPA104 but is less *ts* (26).

quired for full temperature sensitivity affecting in vivo kinase activity.

Secondary structure prediction based upon the method of Chou and Fasman (5) was applied to examine the potential effects of the individual mutations upon the conformation of *p60^{src}* (26). For each of the second strain-specific C-terminal mutations, a significant effect upon secondary structure was predicted (26). The change in secondary structure in the *p60*s of NYD68, NYS72, and NYP104 apparently resulted in stable binding of cellular proteins p50 and p90 to *p60^{src}*. No significant secondary structure change was predicted for the Met-461 mutation alone (26), and NYM72 was wt with respect to complex formation.

The stable binding with the p50-p90 complex observed in hybrid wt-*ts* mutants carrying the second C-terminal mutation did not completely preclude *p60^{src}* membrane association. However, the conformational change induced by the Pro-325 mutation alone (in NYP104) resulted in a decrease in membrane binding as severe as that produced by the concerted effect of both C-terminal mutations in NY68 or NY72 at the nonpermissive temperature. The ability of C-terminal

mutations to impair *p60^{src}* membrane association suggests that stable membrane binding is more complex than mere targeting by an extreme N-terminal membrane-binding domain (9, 12, 29). Stable *p60^{src}*-p50-p90 complex formation may perturb an additional domain involved in the interaction of *p60^{src}* with the membrane (12, 28, 34, 35). Studies with antibodies against synthetic peptides have suggested that complex formation affects both N-terminal (36) and C-terminal (33, 36) regions of *p60^{src}*.

The strain-specific second C-terminal mutation rendered the kinase activity of mutant *p60^{src}*s more susceptible to in vitro heat inactivation than wt *p60^{src}*. In NYD68, NYS72, and NYP104, the kinase activity was as thermolabile as that of the parental *ts* mutants, although no decrease in kinase activity was observed in immune complexes prepared from lysates of infected cells maintained at 41°C. The reason for the discrepancy between these assays is not clear. There may be cellular factors that prevent *p60^{src}* from denaturation and that are lost after cell lysis. The observation of Levinson et al. (23) that the kinase activity of immunoaffinity-purified *ts*NY68 *p60^{src}* was more rapidly heat inactivated than that of immunoaffinity-purified wt *p60^{src}* suggests that increased thermolability is intrinsic to the mutant *p60^{src}*. The increased thermolability correlated with stable complex formation.

Both C-terminal mutations appear to be required to significantly affect in vivo kinase activity, as assayed by 34K protein phosphorylation and *p60^{src}* autophosphorylation, which correlate most strongly with transformation. The decrease in 34K protein phosphorylation relative to that in the wt observed at 37°C in all the mutants except NYM72 most likely reflects stable complex formation, which may affect accessibility to substrates. The defect in autophosphorylation correlates with decreases in both 34K protein phosphorylation and in vitro kinase activity.

The second strain-specific mutation in the kinase domain alters the stability of the *p60^{src}*-p50-p90 complex and may destabilize the kinase activity of the mutant *src* protein, rather severely in the case of PA104. At 41°C, the common Met-461 mutation may interact cooperatively to incapacitate the kinase domain while also altering membrane association. The results of this study suggest that temperature sensitivity involves subtle changes in *p60^{src}* membrane association and kinase activity. The results also suggest that binding to the p50-p90 complex and the thermolability of kinase activity are properties that apparently are more readily susceptible to alteration by mutation than is in vivo *p60^{src}* kinase activity,

FIG. 6. V8 partial proteolytic cleavage analysis of ³²P-labeled mutant *src* proteins. Infected cells maintained at 37°C (lanes a) or 41°C (lanes b) were labeled for 6 h with ³²P_i. Cells were lysed in RIPA buffer, and portions containing equal amounts of total cell protein were immunoprecipitated with TBR serum. *p60^{src}* gel bands were excised and digested with 50 ng of V8 protease during reelectrophoresis on 12.5% SDS-polyacrylamide gels. SRA, SR-RSV-A.

which is altered only by more profound structural alterations.

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