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Medium Tumor Antigen of Polyomavirus Transformation-Defective Mutant NG59 Is Associated with 73-Kilodalton Heat Shock Protein

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Affinity-purified medium T antigen encoded by NG59, a nontransforming mutant of polyomavirus, is specifically associated with a protein of 72,000 daltons (72K protein). Medium T antigens of wild-type polyomavirus and the transformation-competent mutant *dl*8 are not associated with the 72K protein. Instead, they form a complex with another protein of 61,000 daltons. Several lines of evidence suggest that the medium T antigen-associated 72K protein is equivalent to the abundant and constitutive 73K heat shock protein. First, on two-dimensional polyacrylamide gels the 72K protein migrated with the same pI (5.6) as did the 73K heat shock protein. Second, the 72K protein was immunoprecipitable with antibodies against heat shock proteins. Third, when digested with V8 protease, the 72K protein gave rise to the same pattern of fragments as did the 73K heat shock protein.

The transforming protein of polyomavirus, medium T antigen, acquires tyrosine-specific protein kinase activity by forming a complex with pp60$^{c\text{-}src}$, the cellular homolog of the transforming protein of Rous sarcoma virus, pp60$^{v\text{-}src}$ (12, 13). To form a complex with pp60$^{c\text{-}src}$, medium T antigen has to associate with membranes through a stretch of 22 uncharged and hydrophobic amino acids near the C terminus. Forms of medium T antigen which lack this hydrophobic region and therefore cannot associate with membranes have no protein kinase activity and are unable to transform (6, 27). The tyrosine-specific protein kinase activity of pp60$^{c\text{-}src}$ is strongly enhanced by complex formation with medium T antigen (3). It has also been proposed that medium T antigen, rather than enhancing the pp60$^{c\text{-}src}$ kinase activity, might inhibit phosphorylation of pp60$^{c\text{-}src}$ at a tyrosine residue near the C terminus, thereby preventing its inactivation (9, 10). Medium T antigen of the *hrt* mutant NG59 forms a less stable complex with pp60$^{c\text{-}src}$ than does wild-type medium T antigen (1, 2). It has no detectable protein kinase activity and is transformation defective. Thus, the ability of medium T antigen to form a stable complex with pp60$^{c\text{-}src}$ and to activate its protein kinase activity (or to prevent its inactivation) seems to be important for the transforming properties of polyomavirus. As an alternative to the possibility that tyrosine-specific protein kinase activity is essential for transformation, it has been suggested that the medium T antigen-pp60$^{c\text{-}src}$ complex phosphorylates inositol lipids resulting in elevated levels of the second messengers inositol triphosphate and diacylglycerol and in transformation (34). This mechanism seems unlikely because highly purified medium T antigen-pp60$^{c\text{-}src}$ complex is devoid of lipid kinase activity (17).

The interaction between medium T antigen and pp60$^{c\text{-}src}$ is not sufficient for transformation since medium T antigen of the mutant *dl*1015 forms a kinase-active complex with pp60$^{c\text{-}src}$ but is unable to transform (20). Furthermore, medium T antigen with the C-terminal hydrophobic domain of the vesicular stomatitis virus glycoprotein replacing its own C terminus associates with membranes and has protein kinase activity but is defective for transformation (28). Therefore, other properties of medium T antigen besides its ability to associate with membranes and to interact with pp60$^{c\text{-}src}$ must be important for its transforming function. Recently, it was demonstrated that medium T antigen forms specific complexes with two other cellular proteins of 61,000 daltons (61K protein) and 72,000 daltons (72K protein) (16). They were discovered in highly purified preparations of medium T antigen obtained by affinity chromatography with medium T antigen-specific peptide antibodies. The interactions with these two proteins are transformation specific in that medium T antigens of wild-type polyomavirus and the transformation-competent mutant *dl*8 form complexes with the 61K protein but not the 72K protein. Vice versa, the medium T antigens of the transformation-defective *hrt* mutants NG59 and SD15 form complexes with the 72K protein but not with the 61K protein (16). Elucidation of the properties and functions of the 61K and 72K proteins may provide new insight into the transforming mechanism of polyomavirus.

In the present study we demonstrated that the 72K protein, copurifying with the transformation-defective NG59 polyomavirus medium T antigen, is identical to the abundant and constitutive 73K mammalian heat shock protein (29, 35). The 73K heat shock protein is able to bind ATP in vitro and recently has been shown to be identical to a protein involved in the uncoating of clathrin-coated vesicles in an ATP-dependent manner (7, 29, 30, 34).

MATERIALS AND METHODS

Cell culture, virus infection, radiolabeling, and preparation of cell extracts. The procedures for growing and radiolabeling 3T6 cells and preparing cell extracts were carried out as previously described (17).

Antisera and purification of medium T antigen. The production and characterization of the antiserum against the carboxy-terminal peptide Lys-Arg-Ser-Arg-His-Phe (KF) have been published (31). A description of monoclonal antibodies against the peptide Glu-Glu-Glu-Glu-Tyr-Met-

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Pro-Met-Glu (EE) will be published elsewhere. Immunoglobulin G (IgG) from antiserum and hybridoma cell supernatants was purified on protein A-Sepharose as described by Ey et al. (14). Peptide-specific immunoglobulin G was obtained by affinity chromatography on peptide-Sepharose as previously described and was coupled covalently to protein A-Sepharose with dimethyl pimelimidate as described by Schneider et al. (25). An amount of 4 mg of purified immunoglobulin G was coupled per ml of protein A-Sepharose.

Purification of medium T antigen was carried out as previously described (32). Briefly, cell extracts were first incubated for 90 min at 4°C with anti-KF–Sepharose. After being washed, the bound proteins were released from the resin by an excess of KF peptide. The released protein was incubated with EE antibody-Sepharose, and the bound material was released with an excess of EE peptide. For further analysis of the 72K protein on two-dimensional gels, the released material was diluted twofold with gel electrophoresis sample buffer and applied onto a 7.5% polyacrylamide gel. Without fixation, the gel was exposed to X-ray film. The 72K protein was subsequently extracted from the gel with two-dimensional sample buffer and mixed with unlabeled whole-cell extract. For V8 peptide mapping, the medium T antigen-72K protein complex was purified by binding to anti-EE and releasing with EE peptide followed by polyacrylamide gel electrophoresis of the released material. The same procedure was used for purification of the medium T antigen-72K protein complex from sucrose gradient fractions.

Immunoprecipitation of heat shock protein. Antibodies against the mammalian heat shock proteins were prepared as described previously (33). One antiserum recognized only the highly inducible 72K heat shock protein, whereas the other was specific for the family of ATP-binding 72K, 73K, and 80K heat shock proteins (7, 34; Welch, unpublished data). We infected 3T6 cells on a 10-cm-diameter dish with NG59 and labeled them for 3 h in 1.5 ml of methionine-free medium containing 500 μCi of [^{35}S]methionine. Extracts were prepared by lysis in 1 ml of 1% sodium dodecyl sulfate (SDS)–50 mM dithiothreitol–40 mM Tris hydrochloride (pH 6.8)–7.5% glycerol. After boiling for 5 min, the extract was spun at 40,000 rpm for 20 min in a Beckman 50 Ti rotor. A 600-μl volume of extract was diluted 1:10 with RIPA buffer lacking SDS (0.15 M NaCl, 0.01 M sodium phosphate [pH 7.2], 1% deoxycholate, 1% Nonidet P-40, 1 mM dithiothreitol, 50 μM leupeptin) and preincubated with 240 μl of 50% protein A-Sepharose for 10 min at 4°C. After spinning, 3 ml of the supernatant was incubated for 3 h at 4°C with 20 μl of antiserum against heat shock proteins. Immunoprecipitates were bound to protein A-Sepharose, washed with RIPA buffer (0.15 M NaCl, 0.01 M sodium phosphate [pH 7.2], 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM dithiothreitol, 50 μM leupeptin), dissolved in electrophoresis sample buffer, and analyzed on a 7.5% polyacrylamide gel.

Analysis of partial proteolysis. Gel slices containing medium T antigen, 61K protein, and 72K protein were isolated from preparative gels which had not been prepared for fluorography and placed in wells of a 15% polyacrylamide gel. Digestion was with 10 and 50 ng of V8 protease as previously described (8).

One- and two-dimensional polyacrylamide gel electrophoresis. Immunopecipitates were dissolved by boiling in 4% sodium dodecyl sulfate–20 mM dithiothreitol–40% 2-mercaptoethanol–100 mM Tris (pH 6.8)–10% glycerol. Analysis was in gels containing 7.5 or 15% acrylamide and 0.174 or 0.087% bisacrylamide, respectively. Two-dimensional gels used pH 3.5 to 10 isoelectric focusing followed by SDS-polyacrylamide gel electrophoresis on a 7.5% slab gel (15). Visualization of the proteins was done by either staining with silver or PPO treatment followed by autoradiography (21).

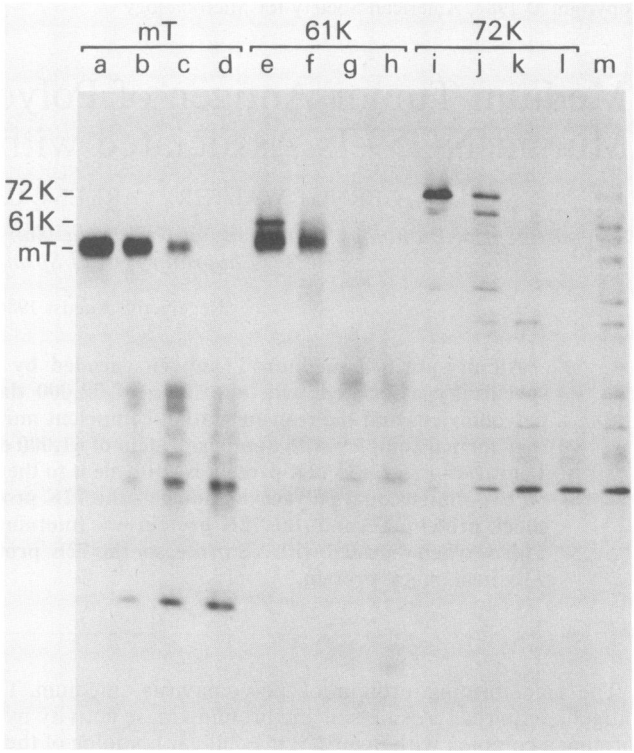

FIG. 1. Partial proteolytic mapping of medium T antigen (mT) and the 61K and 72K proteins. Proteins were purified by affinity chromatography with anti-peptide antibodies and analyzed by SDS-polyacrylamide gel electrophoresis. Gel slices containing medium T antigen and the 61K and 72K proteins were isolated from the preparative gel, placed in the wells of a second gel, and subjected to digestion with 10 (lanes b, f, and j), 50 (lanes c, g, and k), and 200 (lanes d, h, and l) ng of *Staphylococcus aureus* V8 protease. Lanes a, e, and i represent undigested controls. Lane m represents a digest with 50 ng of protease of 72K protein isolated from extract directly without prior precipitation.

RESULTS

The 72K and 61K proteins are unrelated by peptide mapping with V8 protease. To examine the possibility that the 72K protein might be the precursor or a modified form of the 61K protein, both proteins were compared by peptide mapping. We infected 3T6 cells with the transformation-competent mutant *dl*8 and the nontransforming *hrt* mutant NG59. The respective medium T antigens were immunoprecipitated from infected-cell extracts with antibodies against a synthetic peptide corresponding to an internal region of medium T antigen (see Materials and Methods). The 61K protein, coprecipitating with *dl*8 medium T antigen, and the 72K protein, coprecipitating with NG59 medium T antigen, were extracted from the polyacrylamide gels and digested with V8 protease. The patterns of proteolytic fragments of these two proteins looked very different (Fig. 1), suggesting that the 72K protein is not a precursor or a simple modification of the 61K protein. We also demonstrated (Fig. 1) that the V8 map of *dl*8 medium T antigen is different from

those of the 72K and 61K proteins, indicating that medium T antigen is unrelated to the associated proteins. A strong band of 72,000 daltons was also detected when cytoplasmic extracts from wild-type- or NG59-infected, as well as mock-infected, cells were analyzed directly on polyacrylamide gels without prior immunoprecipitation. This protein gave rise to the same V8 map as did purified 72K protein (Fig. 1), demonstrating that the 72K protein is an abundant cellular protein.

The 72K protein migrates like heat shock protein on two-dimensional polyacrylamide gels. A first clue as to the possible nature of the 72K protein came from its behavior on two-dimensional polyacrylamide gels. NG59-infected cells were labeled with [^{35}S]methionine, and medium T antigen was purified by affinity chromatography with two peptide antibodies followed by polyacrylamide gel electrophoresis (see Materials and Methods). The purified protein was mixed with unlabeled whole-cell extract from uninfected 3T6 cells and analyzed by two-dimensional polyacrylamide gel electrophoresis. The location of the radioactive 72K protein was determined by autoradiography of the stained gel. The 72K protein migrated in the gel with an apparent isoelectric point of approximately 5.6 (Fig. 2). Its apparent size, isoelectric point, and migration relative to other proteins (e.g., bovine serum albumin and actin) appeared very similar to those described for the constitutive and abundant mammalian 73K heat shock protein (35).

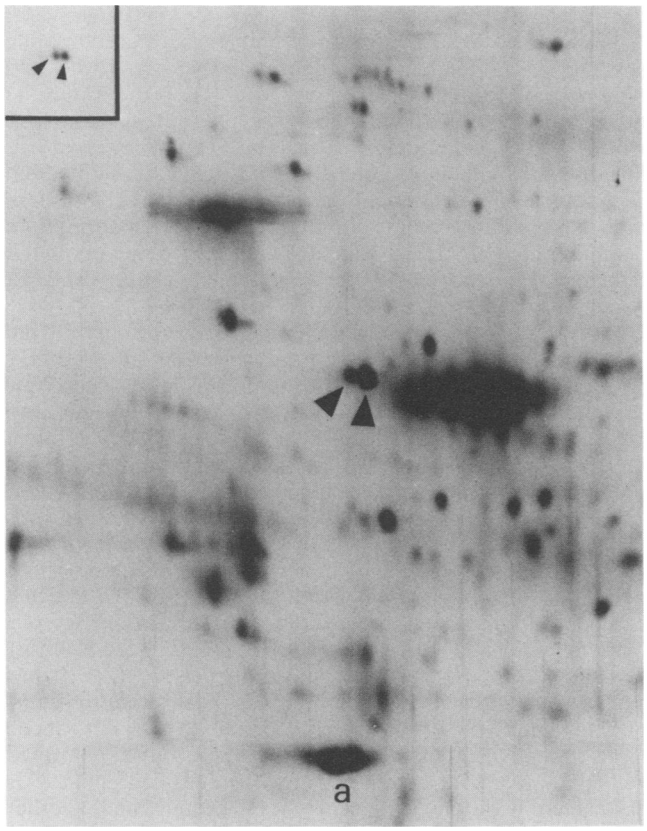

FIG. 2. Identification of the 72K protein by two-dimensional gel analysis. ^{35}S-labeled 72K protein was purified by affinity chromatography and gel electrophoresis and mixed with unlabeled whole-cell extract from 3T6 cells. Two-dimensional polyacrylamide gel electrophoresis was carried out as described in Materials and Methods. The gel was stained with silver and subsequently treated with PPO followed by autoradiography. The gel is shown with the acidic side to the left. The large arrows indicate the position of silver-stained 72K proteins which were radioactive as shown by fluorography. The insert shows a section of the fluorogram containing the radioactive 72K protein. The letter a indicates the position of actin. The heavily stained protein to the right of the 72K protein is presumably bovine serum albumin, a contaminant in the whole-cell extract from the growth medium. This protein is absent from fluorograms of two-dimensional gels of [^{35}S]methionine-labeled whole-cell extract.

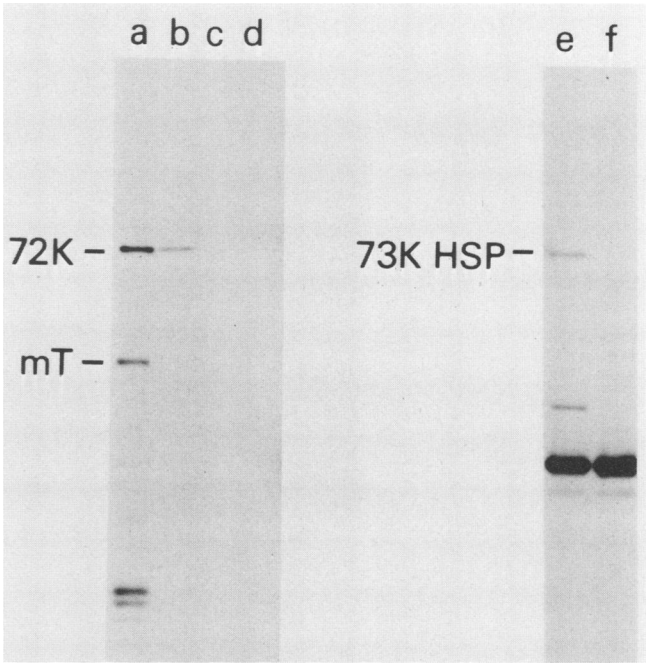

FIG. 3. Immunoprecipitation of the 72K protein and the 73K heat shock protein (73K HSP) with antibodies to heat shock proteins. Lanes: a, [^{35}S]methionine-labeled 72K protein-medium T antigen (mT) complex was purified by binding to anti-EE and released with EE peptide; b, immunopecipitate of material shown in lane a with antiserum to the 72K, 73K, and 80K heat shock proteins; c, immunoprecipitate of material shown in lane a with antiserum specific for the 72K heat shock protein; d, immunoprecipitate with control serum; e, immunoprecipitate of the 73K heat shock protein from whole-cell extract with antiserum against the 72K, 73K, and 80K heat shock proteins; f, precipitation from whole-cell extract with control serum.

Identity of the 72K protein and the 73K heat shock protein. Conclusive evidence that the 72K protein associated with medium T antigen and the 73K heat shock protein are identical or closely related was obtained by immunoprecipitating purified 72K protein with heat shock protein-specific antibodies, and by comparative V8 protease peptide mapping. Two different kinds of antibodies were available, one recognizing the 72K, 73K, and 80K heat shock proteins (i.e., the family of proteins which bind ATP [7]), the other directed against the 72K heat shock protein only. The material used for immunoprecipitation was 72K protein copurified with medium T antigen (Fig. 3, lane a). The antiserum against the 72K, 73K, and 80K heat shock proteins precipitated the 72K protein very well (Fig. 3, lane b), whereas the antibody specific for the 72K heat shock protein precipitated only trace amounts of the 72K protein (lane c). This result suggested that the 72K protein is most likely the 73K heat shock protein. This suggestion was confirmed by a comparison of the V8 peptide maps of the 73K heat shock

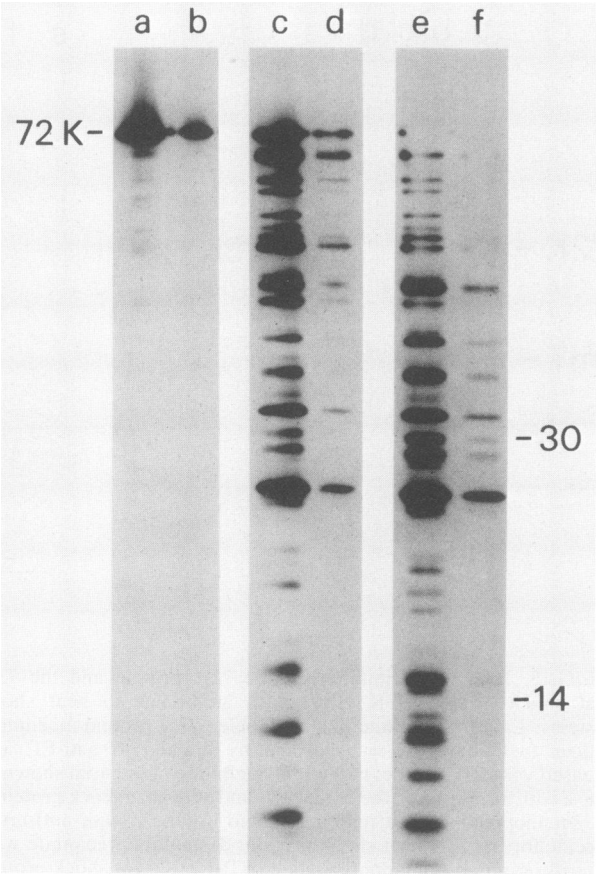

FIG. 4. Partial proteolytic mapping of the 72K protein and the 73K heat shock protein. [^{35}S]methionine-labeled 72K protein was isolated from NG59-infected 3T6 cells by affinity chromatography and polyacrylamide gel electrophoresis, and [^{35}S]methionine-labeled heat shock protein was obtained from whole-cell extract by immunoprecipitation with antiserum to the 72K, 73K, and 80K heat shock proteins. Both proteins were digested with 10 (lanes c and d) or 50 (lanes e and f) ng of *S. aureus* V8 protease. Lanes a and b, samples with undigested proteins; a, c, and e, the 72K protein; b, d, and f, the 73K heat shock protein.

protein isolated by immunoprecipitation from whole-cell extracts by using heat shock protein-specific antiserum (see Materials and Methods) with affinity-purified medium T antigen-associated 72K protein. Figure 3, lanes e and a, respectively, show the immunoprecipitated 73K heat shock protein and the affinity-purified 72K protein, which were used for V8 peptide mapping. The V8 peptide maps of the two independently isolated proteins are shown in Fig. 4. They are virtually indistinguishable, suggesting strongly that the 72K protein, associated with medium T antigen of NG59, is in fact the 73K heat shock protein. The strongest band in Fig. 3, lane e, most likely represents the major capsid protein VP1 and can be considered nonspecific since it was also seen in precipitates with control serum (Fig. 3, lane f).

To demonstrate directly that the 72K protein (or 73K heat shock protein) forms a complex with NG59 medium T antigen, cytoplasmic extract from NG59-infected 3T6 cells was analyzed on a 5 to 20% glycerol gradient. Medium T antigen was purified from each fraction by affinity chromatography (16). It was found that the 72K protein cosedimented with medium T antigen, indicating that the two proteins formed a complex (data not shown).

DISCUSSION

A characteristic property of medium T antigen is its ability to associate with other proteins. Three such proteins have been characterized so far: pp60$^{c\text{-}src}$, 61K protein, and 72K protein, the latter now being identified as the 73K heat shock protein. pp60$^{c\text{-}src}$ was discovered in a complex with medium T antigen because of its intrinsic protein kinase activity and the availability of specific antibodies (12, 13). It would most likely not have been detected by metabolic labeling because only a small fraction of the total cellular pp60$^{c\text{-}src}$ is associated with medium T antigen (3). The 61K and 72K proteins, for which functional assays do not exist, were identified because they copurified with medium T antigen and were present in large enough quantities to be detectable by labeling experiments (16). It is possible that still other proteins are specifically associated with medium T antigen. We have consistently observed three minor proteins of 88K, 37K, and 31K which copurified with the medium T antigen-61K protein complex (17). They have not been further characterized.

The interactions of medium T antigen show remarkable specificity. On one hand, medium T antigens from the wild type and *dl*8 form complexes with the 61K protein. On the other hand, medium T antigens from *hrt* mutants associate with the 73K heat shock protein. The 61K protein behaves like pp60$^{c\text{-}src}$, which also binds to wild-type and *dl*8 medium T antigens. It was recently found that the 61K protein associates with medium T antigens of the transformation-defective mutants *dl*23 and *dl*1015 (T. Grussenmeyer, K. H. Scheidtmann, and G. Walter, unpublished data), both of which also bind pp60$^{c\text{-}src}$.

It is an open question whether the 61K protein and the 73K heat shock protein bind to the same site on medium T antigen. It is possible that the mutation in NG59 decreases the affinity of medium T antigen for the 61K protein and increases its affinity for the 73K heat shock protein. To test these possibilities, one would have to mix the purified proteins in vitro and study their interaction.

The finding that the 73K heat shock protein associates with medium T antigen raises the possibility that it plays a role in growth control. Two other proteins, p53 and pp60$^{v\text{-}src}$, both involved in regulation of cell proliferation, form specific complexes with heat shock proteins (4, 18, 22, 24, 26). p53 binds to 72K and 73K heat shock proteins in cells transformed by *ras* and p53 and overproducing p53 (24). It has been suggested that the 72K and 73K heat shock proteins might stabilize p53 in these cells, similar to simian virus 40 large T antigen stabilizing p53 in simian virus 40-transformed cells (23). A small fraction of newly synthesized pp60$^{v\text{-}src}$ is associated with the 90K heat shock protein and another protein called p50. This complexed form of pp60$^{v\text{-}src}$ is located in the cytoplasm and is inactive as a protein kinase. As the complex reaches the plasma membrane, it dissociates and pp60$^{v\text{-}src}$ is deposited in the plasma membrane, where it becomes active as tyrosine kinase (5, 11). It has been suggested, among other possibilities, that the 90K heat shock protein might be a negative regulator of kinase activity, like the regulatory subunit of cyclic AMP-dependent kinase. One can ask whether the absence of kinase activity in the NG59 medium T antigen-pp60$^{c\text{-}src}$ complex is mediated by the 73K heat shock protein. It is possible that medium T antigen, pp60$^{c\text{-}src}$, and the 73K heat shock protein form a triple complex in which the 73K heat shock protein suppresses the activity of pp60$^{c\text{-}src}$. Evidence for such a complex has been obtained recently (17). Alternatively, NG59 me-

dium T might not be able to prevent inactivation of the associated protein kinase by phosphorylation of the C-terminal tyrosine 527 residue (9).

Our finding that 73K heat shock protein binds only to the mutant medium T antigen and little, if at all, to the wild-type protein is reminiscent of the fact that the 90K heat shock protein associates much more strongly with $pp60^{v\text{-}src}$ from temperature-sensitive mutants than from the wild type. As shown by Brugge et al. (4, 5), 95% of $pp60^{v\text{-}src}$ in cells transformed with temperature-sensitive mutants of $pp60^{v\text{-}src}$ are associated with the 90K heat shock protein, as compared with only 5% in wild-type-infected cells. Similarly, it was mentioned by Pinhasi-Kimhi et al. (24) that the 72K and 73K heat shock proteins have a higher affinity for the mutant p53 than for the wild-type protein. Perhaps in all three cases the mutant proteins have a more hydrophobic surface area than the corresponding wild-type proteins and, therefore, have a higher affinity for heat shock proteins. This view is consistent with a recent suggestion that the function of heat shock proteins in the cell nucleus of heat-shocked cells is to dissolve, in an ATP-dependent fashion, aggregates of denatured proteins formed by "improper" hydrophobic interactions (19). According to this model, heat shock proteins have the potential of turning into ATPases after making contact with a proper substrate (7, 30). It should be interesting to study whether the medium T antigen-73K heat shock protein complex has ATPase activity.

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