Association of a Cellular Heat Shock Protein with Simian Virus 40 Large T Antigen in Transformed Cells

EARL T. SAWAI AND JANET S. BUTEL*

Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030

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The viral oncoprotein of simian virus 40, large T antigen (T-ag), is essential for viral replication and cellular transformation. To understand the mechanisms by which T-ag mediates its multifunctional properties, it is important to identify the cellular targets with which it interacts. A cellular protein of 73 kilodaltons (p73) which specifically associates with T-ag in simian virus 40-transformed BALB/c 3T3E cells has been identified. The binding of p73 to T-ag was demonstrated by coimmunoprecipitation analyses using polyclonal and monoclonal antibodies specific for T-ag. The interaction of p73 with T-ag was independent of T-ag complex formation with the cellular protein p53. Partial V8 protease cleavage maps for p73 and the cellular heat shock protein hsp70 were identical. Immunoblot analyses indicated that p73 complexed to T-ag was antigenically related to hsp70. T-ag deletion mutants were constructed that remove internal, amino-terminal, and carboxy-terminal sequences. These mutants mapped the p73 binding domain to the amino terminus of T-ag. The specific dissociation of p73 from the p73/T-ag complex was mediated by ATP; GTP, CTP, and UTP were also utilized as substrates. These characteristics suggest that p73 may be a member of the hsp70 family of heat shock proteins. The biologic significance of p73/T-ag complex formation has yet to be determined.

Simian virus 40 (SV40) large T antigen (T-ag) is a multifunctional protein that is essential for viral replication and responsible for morphologic transformation of rodent cells (for reviews, see references 6, 50, 67, and 79). Little is known about the mechanisms by which T-ag mediates those multiple biologic properties. Therefore, it is necessary to identify the cellular components with which T-ag may interact. T-ag is known to specifically bind the cellular protein p53 (67) and DNA polymerase alpha (20), and a recent report indicated that T-ag can associate with the retinoblastoma (Rb) susceptibility gene product (16). We present evidence here that T-ag also interacts with a cellular heat shock protein, hsp70.

hsp70 is a member of an evolutionarily highly conserved family of proteins whose expression is induced by heat shock or other physiologic stresses; some members of the family are constitutively expressed in cells (for reviews, see references 13, 54, and 74). The synthesis of heat shock proteins may be induced by viral infection (12, 21, 35, 37, 47-49, 55, 62), and elevated expression of the major mammalian heat shock protein hsp70 has been noted after infection by adenovirus, polyomavirus, SV40, and herpes simplex virus type 2 (37, 48, 62). Members of the hsp70 family of proteins also are expressed in many virus-transformed cells (37, 62, 65, 80; M. Y. Lim and H. R. Bose, personal communication). The early gene products of the DNA tumor viruses (e.g., SV40 T-ag and the adenovirus E1A 13S gene product) are responsible for induction of hsp70, and the immediate-early gene products of herpes simplex virus have been shown to induce hsp70 synthesis in human cells (37, 63, 89). The large T-ag of polyomavirus reportedly binds to two specific pentanucleotide sequences in the human hsp70 promoter (38), but binding does not specifically correlate with hsp70 induction.

Whereas a functional role for hsp70 in transformed cells has not been delineated, clues to its function may be obtained from those proteins with which it interacts. hsp70

and its constitutively expressed homolog, hsc70, reportedly bind cellular protein p53 in transformed cells that overexpress p53 (19, 30, 66, 81). The domains involved in the interaction between hsc70 and p53 appear to be evolutionarily highly conserved, because p53 can bind the *Escherichia coli* homolog of hsp70, the *dnaK* gene product (1, 10). As functional conservation of enzymatic activity was observed for the bacterial heat shock protein (10), homologous cellular pathways may exist in eucaryotic systems that utilize hsp70-like proteins.

The specific association of hsp70 with viral oncogene products has been noted, including hsp70 binding to the nontransforming polyomavirus middle T-ag mutant NG59 (84), to adenovirus E1A (88), and to the *v-rel* oncogene protein (Lim and Bose, personal communication). The pp60^{src} family of proteins associate with a different heat shock protein, hsp90, in complex with yet another cellular protein of 50 kilodaltons (kDa) (55, 91). Similarly, hsp90 has been reported to bind the cellular glucocorticoid receptor (72, 91).

We present evidence that SV40 T-ag specifically interacts with a heat shock-like protein of 73 kDa (p73) that possesses a broad nucleoside triphosphate-binding activity. The interaction is independent of T-ag/p53 complex formation. Using T-ag deletion mutants, we have mapped the p73 binding site to the amino terminus of the T-ag molecule.

MATERIALS AND METHODS

Cells and cell culture. The BALB/c 3T3E cell line was a gift from W. W. Brockman (4). The wild-type (WT) virus transformant, the mKSA-Asc cell line, was derived from BALB/c mouse kidney cells (39). The transformed BALB/c 3T3E cell lines (ST300, ST510, and ST803) constitutively express SV40 T-ag deletion mutants SVd1423, SVd1425, and SVd1428, respectively (see below). The GM847 cell line (32) was obtained from the Institute for Medical Research, Camden, N.J. The hybridoma cell line m73 was provided by E. Harlow (27). All cells, with the exception of the hybridoma cell line, were grown at 37°C in Dulbecco minimal essential

* Corresponding author.

medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum (GIBCO), 0.3% sodium bicarbonate, and 25 µg of gentamicin sulfate (Schering Corp., Bloomfield, N.Y.) per ml. The m73 cells were grown in HY-15 hybridoma media: Dulbecco minimal essential medium supplemented with 20% fetal calf serum, 10% NCTC-109 (GIBCO), 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 0.3% sodium bicarbonate, 4 mM L-glutamine, 0.125 mg of glutathione, 0.15 mg of oxaloacetic acid, 50 µg of pyruvate, 8 µg of insulin, and 8 µg of transferrin per ml (all from Sigma Chemical Co., St. Louis, Mo.), 50 µM 2-mercaptoethanol (Bio-Rad Laboratories, Richmond, Calif.), and 25 µg of gentamicin sulfate per ml.

Plasmids. Plasmid pBSV-1 contains an intact copy of a WT SV40 genome inserted into the *Bam*HI site of pBR322 (45). Plasmids pSVdl423, pSVdl425, and pSVdl428 are deletion mutants of the SV40 T-ag gene derived from pBSV-1 (Sawai and Butel, manuscript in preparation). Plasmid p262Neo, which contains the neomycin resistance gene inserted into a murine leukemia retroviral vector (4a), was a generous gift from M. Scott (University of California at San Francisco) via L. Donehower (Baylor College of Medicine). Plasmids were propagated in *E. coli* HB101 or GM161 (3) in the presence of 100 µg of ampicillin per ml. Chloramphenicol-amplified plasmid DNA was purified by using a modification of the alkaline extraction procedure of Birnboim (2) and cesium chloride density gradient centrifugation.

Antisera and monoclonal antibodies. Hamster ascites fluid (HAF) from SV40 tumor-bearing animals was prepared as previously described (44). The T-ag-specific hybridomas PAb402, PAb405, PAb414, PAb416, PAb419, PAb423, and PAb430 and the p53-specific PAb421 hybridoma were provided by E. Harlow (26). The anti-T-ag hybridoma PAb204 was a gift from R. Tjian (9, 42). T-ag-specific hybridomas PAb100 and PAb101 were provided by E. Gurney (25), and PAb602 was received from L. Gooding (23). Whereas monoclonal antibodies PAb100, PAb402, PAb204, PAb416, and PAb602 recognize internal antigenic determinants, monoclonal antibodies PAb419 and PAb430 react with extreme amino-terminal sites and monoclonal antibodies PAb405, PAb423, and PAb101 recognize extreme carboxy-terminal T-ag epitopes. The anti-p53 hybridoma 200.47 was obtained from A. DeLeo (18). The monoclonal antibodies were used as ascites fluids or as immunoglobulin G (IgG) concentrates from tissue culture media. Normal hamster serum (Biotrol, Madison, Wis.) and the anti-adenovirus 5 E1A-specific monoclonal antibody m73 were used as controls for immunoprecipitation analyses. The mouse anti-human hsp72/73 monoclonal antibody N27F3-4 (81) and purified hsp70 from HeLa cells (87) were generous gifts from W. Welch.

Transfection of BALB/c 3T3E cells. BALB/c 3T3E cells were transfected by using a modification of a previously described method (46). Briefly, BALB/c 3T3E cells that were 70% confluent on 60-mm petri dishes were cotransfected with calcium phosphate precipitates containing 5 µg of SV40 plasmid DNA and 1.5 µg of p262Neo. At 24 h after application of the calcium phosphate DNA precipitates, media were changed to 10% Dulbecco minimal essential medium containing 1 mg of gentamicin (G418; GIBCO) per ml. Individual G418-resistant colonies were selected after 3 weeks and were amplified by sequential passage. Amplified colonies were screened for T-ag expression by indirect immunofluorescence (5) using HAF and fluorescein-conjugated rabbit anti-hamster IgG (Miles Scientific Div., Miles Laboratories, Inc., Naperville, Ill.). Cell lines exhibiting

strong T-ag fluorescence were then characterized by immunoprecipitation using T-ag-specific serum and monoclonal antibodies and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Metabolic labeling, extraction, immunoprecipitation, and SDS-PAGE. Subconfluent (80%) monolayers were washed three times with warm Tris-buffered saline and were pulse-labeled for 3 h with [³⁵S]methionine (ICN Radiochemicals, Irvine, Calif.) in methionine-free Eagle minimal essential media (GIBCO) supplemented with 2% dialyzed fetal calf serum. Cells were extracted as previously described (34). Cell extracts were preadsorbed with Formalin-inactivated, heat-killed *Staphylococcus aureus* Cowan I (36) to reduce nonspecific binding of cellular proteins to *S. aureus* Cowan I. Sequential immunoprecipitations were performed (34) by using preincubations of either normal hamster serum or m73 IgG, followed by immunoprecipitations with anti-p53 or anti-T-ag antibodies. Immune complexes were analyzed by SDS-PAGE as previously described (34), except that 10 and 12% acrylamide gels were used.

ATP release assay. The ATP release assay for p73 was a modification of the procedure described by Munro and Pelham (61). All manipulations were performed at 4°C. Briefly, immunoprecipitates were washed three times with ATP release assay buffer (25 mM Tris hydrochloride [pH 7.4], 140 mM NaCl, 1 mM MgCl₂). The immunoprecipitates were incubated for 1 h in ATP release assay buffer containing 1 mM ATP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) or 1 mM of one of the following nucleotides: dATP, ADP, AMP, GTP, adenylyl-imidodiphosphate (AMP-PNP), ATP-γ-S, guanylyl-imidodiphosphate (GMP-PNP), guanylyl(β,γ-methylene)-diphosphonate (GMP-PCP), CTP, or UTP (Boehringer Mannheim). The immunoprecipitates were centrifuged for 1 min at 14,000 rpm in a microcentrifuge (Fisher Scientific Co., Pittsburgh, Pa.). The supernatant and pellet were suspended in disruption buffer and were heated at 100°C for 10 min. After centrifugation for 5 min, the supernatant was analyzed by SDS-PAGE.

V8 protease mapping. One-dimensional partial protease mapping was performed as described (11) using *S. aureus* V8 protease (Sigma). After electrophoresis, the gels were impregnated with Autofluor (National Diagnostics, Manville, N.J.), dried, and fluorographed.

One-dimensional isoelectric focusing. Isoelectric focusing (IEF) was performed as previously described (76). Briefly, [³⁵S]methionine-labeled p73 was released from T-ag immune complexes by incubating immunoprecipitates with ATP as described above. Supernatants containing released p73 were dialyzed against 30 volumes of Tris-buffered saline and were concentrated by using Centricon 30 microconcentrators (Amicon Corp., Danvers, Mass.). IEF was performed in 2-mm acrylamide gels containing 2% ampholytes (pH range, 3.5 to 10) and 0.1% Tween 20. The electrode wicks were saturated with 1.0 N NaOH (cathode) and 1.0 M H₃PO₄ (anode), and the samples were applied to the gel surface by using paper applicators (3 MM; Whatman, Inc., Clifton, N.J.). Electrophoresis was carried out for 2 h at a final wattage of 30 using a Multiphor apparatus (LKB Instruments, Inc., Rockville, Md.). Measurements of the pH gradient were made with a surface pH probe immediately after electrofocusing. pH determinations were confirmed by comparison with the location of IEF standards having known isoelectric points (pI). Standards included soy bean trypsin inhibitor (pI, 4.55), β-lactoglobulin (pI, 5.2), bovine carbonic anhydrase (pI, 5.85), human carbonic anhydrase B (pI, 6.55), horse myoglobin (pI, 6.76 and 7.16), trypsinogen

FIG. 1. Structures of newly constructed in-phase deletion mutants of SV40 T-ag. Mutant designations are shown on the left; BALB/c 3T3E cell lines constitutively expressing the mutant T-ag are indicated on the right. Numbers below the open bars indicate the authentic amino acid residues contained in each mutant T-ag; no stretches of extraneous amino acids are present in any of the deleted T-ag polypeptides. The positions of the first and second exons and the nuclear transport (NT) signal in WT T-ag are indicated at the top.

(pI, 9.3), and cytochrome *c* (pI, 10.25). Gels were fixed with 20% trichloroacetic acid for 20 min, stained with Coomassie blue, dried, and autoradiographed.

Immunoblot analysis. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose filters (82), and the filters were processed as previously described (78). Mouse anti-human hsp72/73 monoclonal antibody N27F3-4 was used as the first antibody; after three washes with blocking buffer, a second antibody, rabbit anti-mouse IgG (Organon Teknika, Malvern, Pa.), was added. Blocking buffer washes were followed by the addition of ^{125} I-labeled *S. aureus* protein A as previously described (78). Radioautography was performed using dried nitrocellulose filters.

RESULTS

Construction of SV40 mutants and establishment of mouse cell lines constitutively expressing mutant T-ag. As an approach to delineating the biologic functioning of SV40 T-ag, several in-phase deletion mutants were designed. Constructs were generated that deleted amino-terminal, carboxy-terminal, or internal sequences of T-ag (Fig. 1). Nomenclature designations for the T-ag deletion mutants were assigned according to the conventions described by Robb et al. (68). Stable BALB/c 3T3E cell lines that constitutively express the respective mutant T-ag were established by calcium phosphate cotransfection with a dominant selectable marker plasmid, p262Neo, and subsequent selection for G418-resistant transformants. The mutant T-ag were of the predicted size and accumulated to readily detectable levels in the expressor cell lines (Fig. 2, panels A and B).

Coimmunoprecipitation of a 73-kDa cellular protein from mutant T-ag-expressing cell lines. During immunoprecipitation analyses of internal in-phase deletion mutants of T-ag expressed in mouse cells, a prominently labeled protein of approximately 73 kDa (p73) appeared to be coimmunoprecipitated by the polyclonal anti-T-ag serum HAF (Fig. 2A). p73 was coimmunoprecipitated with the deletion mutant T-ag encoded by *dl428* and with WT T-ag, but it was not recovered from the untransformed BALB/c 3T3E cells (Fig. 2A). WT T-ag appeared to have a lower affinity for p73 than did the *dl428* deletion mutant. Interestingly, detectable amounts of p73 were not coimmunoprecipitated with the *dl423* mutant protein, which lacks the amino-terminal 108 amino acids of T-ag, from ST300 cells (Fig. 2A). In contrast, the ST510 cell line constitutively expressed the *dl425* truncated mutant that contains the amino-terminal 178 amino

FIG. 2. Immunoprecipitation of WT and mutant T-ag from T-ag-transformed BALB/c 3T3E cells. (A) The cell lines mKSA-Asc (WT), ST803 (803), ST300 (300), and BALB/c 3T3E (3T3) were metabolically labeled with [^{35} S]methionine and were extracted with extraction buffer containing 1% Nonidet P-40. Clarified extracts were first immunoprecipitated with the control monoclonal antibody m73 (N). Immune complexes were adsorbed with heat-inactivated, Formalin-fixed *S. aureus* Cowan I. After the first immunoprecipitation, the extracts were equally divided, and each was immunoprecipitated with T-ag-specific serum (HAF) or monoclonal antibodies (PAb430 or PAb602) as described above. Proteins were eluted from the bacterial pellets with SDS disruption buffer and were analyzed by SDS-PAGE on 10% polyacrylamide gels. Protein bands were visualized by autoradiography. (B) BALB/c 3T3E cells expressing the *dl425* mutant T-ag (ST510) were metabolically labeled, and cellular extracts were prepared as described for panel A. Immunoprecipitation of *dl425* T-ag (T) was performed as described for panel A, first using the control monoclonal antibody m73 (N), followed by a T-ag-specific monoclonal PAb100 (100), PAb430 (430), or PAb416 (416). Immune complexes were recovered from the *S. aureus* Cowan I pellets as described above and were resolved by SDS-PAGE on 12% polyacrylamide gels. Molecular sizes (MW) (in kilodaltons) are indicated on the left, and the positions of T-ag (T), t-ag (t), and p73 are indicated on the right.

FIG. 3. Coimmunoprecipitation of p73 with a T-ag mutant was specific for T-ag. ST803 cells expressing the T-ag deletion mutant SVd428 were labeled with [^{35} S]methionine and were extracted as described in the legend to Fig. 2. Cell extracts were sequentially immunoprecipitated, first with the control monoclonal antibody m73 (N), followed by immunoprecipitation with polyclonal anti-T-ag sera (HAF) or one of the following T-ag-specific monoclonal antibodies: PAb100, PAb101, PAb204, PAb402, PAb405, PAb414, PAb416, PAb419, PAb423, PAb430, or PAb602. The proteins were separated by SDS-PAGE on 10% polyacrylamide gels. The positions of T-ag (T), p73, and p53 are indicated on the right. Molecular sizes (in kilodaltons) are indicated on the left.

acids of T-ag. The *dl425* mutant T-ag was recognized by amino-terminal-specific monoclonal antibodies PAb430 and PAb416 (26) but not by monoclonal antibody PAb100, which recognizes an internal epitope of T-ag (25) (Fig. 2B). As expected, the PAb430 antibody recognized both the mutant T-ag fragment and small t antigen (t-ag), whereas only mutant T-ag was recognized by the PAb416 monoclonal antibody (Fig. 2B). p73 was coimmunoprecipitated with the truncated T-ag. A comparable amount of p73 was recovered regardless of whether t-ag was coprecipitated, indicating that the p73 interaction was specific for T-ag and not for sequences in common with t-ag.

The presence of the prominent 73-kDa band in T-ag precipitates could be explained in several ways in addition to a specific interaction of the protein with T-ag. First, since the polyclonal HAF sera possessed reactivities against T-ag, t-ag, and p53, it was possible that anti-p73 antibodies also were present. Second, because of the multiple antibodies in HAF, p73 may have been coimmunoprecipitated due to an interaction with t-ag or p53. Third, the recovery of p73 from cellular extracts may have been nonspecific, due either to adsorption of p73 to *S. aureus* Cowan I or to a fortuitous cross-reaction between T-ag-specific antibodies and p73. Finally, p73 may have been a degradation product of T-ag. These alternative explanations were ruled out in the following series of experiments.

Immunoprecipitation of p73 from cell extracts was dependent on complex formation with T-ag. To determine whether immunoprecipitation of p73 was specific for the presence of

T-ag and not due to the existence of anti-p73 antibodies in the polyclonal serum, various monoclonal antibodies were tested for the ability to coimmunoprecipitate p73 along with T-ag. A battery of monoclonal antibodies specific for internal, amino-terminal, or carboxy-terminal epitopes on T-ag all coimmunoprecipitated p73 (Fig. 3). The only exception was PAb602, which recognizes an internal T-ag determinant (23). These results indicate that the presence of p73 in immune complexes was dependent upon the coincident presence of T-ag.

It has been established that SV40 t-ag specifically interacts with two cellular proteins of 56 and 32 kDa (70, 71). The possibility that t-ag may have been binding p73 was ruled out by the ability of monoclonal antibodies specific for the carboxy terminus of T-ag (which are not in common with t-ag sequences) to coimmunoprecipitate p73 (Fig. 3).

Because recent reports have indicated that p53 may bind hsp70 or hsc70 or both (19, 30, 66, 81), we investigated whether p73 was coprecipitated due to an association with p53 that was, in turn, complexed to T-ag. This possibility was eliminated when we performed sequential immunoprecipitations on extracts of ST803 cells. An anti-p53 monoclonal antibody (PAb421) was used initially, followed by treatment of the cell extracts with an anti-T-ag monoclonal antibody (PAb419). The first immunoprecipitation with PAb421 cleared the extracts of p53, as well as some complexed T-ag; the second immunoprecipitation revealed T-ag and associated p73 in the absence of p53 (Fig. 4).

These observations were confirmed by sucrose gradient

FIG. 4. Specific release of p73 from T-ag immunoprecipitates by incubation in the presence of ATP. Normal BALB/c 3T3E cells and cells transformed by *dl425* (510) and *dl428* (803) were metabolically labeled and extracted as described in Fig. 2. A sequential immunoprecipitation analysis was performed using antisera in the following order: normal hamster serum (NHS), anti-p53 PAb421 (421), and anti-T-ag PAb419 (419). Each immunoprecipitate was suspended in a solution containing 25 mM Tris (pH 7.4), 140 mM NaCl, 1 mM MgCl₂, and 1 mM ATP and was incubated for 1 h at 4°C. The bacteria were pelleted, and the pellet (P) and supernatant (S) were analyzed by SDS-PAGE on 12% polyacrylamide gels. The positions of T-ag (T), p73, and p53 are indicated on the right. Molecular sizes (in kilodaltons) are indicated on the left. Note that p73 was released into the supernatants from some of the precipitates during the incubation period.

sedimentation analyses of similar cell extracts that had been cleared of p53 by treatment with monoclonal antibodies PAb421 and 200.47 (Fig. 5). The gradient fractions were immunoprecipitated with the T-ag-specific antibody PAb419. p73 cofractionated with T-ag in the middle of the 5 to 20% sucrose gradient. Finally, the deletion mutants clearly separated complex formation of p73 with T-ag from the interaction of p53 with T-ag. Although the *dl423* mutant T-ag bound p53 to the same extent as did WT T-ag (Fig. 2), we failed to detect p73 binding to that mutant. Conversely, the *dl425* mutant protein, which contains the amino-terminal 178 amino acids of T-ag and lacks the p53-binding domain, bound p73 (Fig. 2B and 4).

The recovery of p73 appeared to be due to an association with T-ag rather than to a fortuitous cross-reactivity with T-ag-specific antibodies. Eleven different monoclonal antibodies directed against distinct antigenic determinants on T-ag all coprecipitated p73; it is extremely unlikely that an unrelated protein would share so many epitopes. Moreover, p73 was not among those cellular proteins previously described to cross-react with selected T-ag-specific monoclonal antibodies (15, 42, 43).

p73 was not a proteolytic degradation product of T-ag. Comparisons of partial protease maps of p73 with those for

T-ag and p53 indicated that p73 is different from those two proteins (data not shown). Also, unlike T-ag, p73 was not phosphorylated (data not shown). One would expect a proteolytic degradation fragment of that size to be phosphorylated at either the amino- or carboxy-terminal T-ag sites (73).

Relationship of p73 to heat shock proteins. Because p73 is similar in size to known heat shock proteins, we determined whether p73 possesses properties similar to those described for members of the hsp70 family of proteins (64). An ATP release assay was performed (Fig. 4) because heat shock proteins characteristically possess intrinsic ATPase activity. ATP was incubated with immunoprecipitates obtained by sequential treatment of cell lysates with monoclonal antibodies, and the immune complexes (pellets) and supernatants were analyzed by SDS-PAGE. After immune antibody-bound complexes containing T-ag and p73 were incubated with ATP, p73 was found to be released into the supernatants (Fig. 4). p73 was detected in the supernatants from PAb421 and PAb419 precipitates from ST803 cells and from the PAb419 precipitate from ST510 cells, whereas release was not observed from the control normal hamster serum precipitates. These results indicate the presence of a release activity mediated by ATP in the precipitates containing T-ag

FIG. 5. p73 cosedimented with mutant T-ag in sucrose gradients. ST803 cells expressing the T-ag deletion mutant SVd428 were labeled with [^{35} S]methionine and extracted as described in the legend to Fig. 2. Cell extracts were sequentially immunoprecipitated, first with the control monoclonal antibody m73 (N), followed by immunoprecipitation using the p53-specific monoclonal antibodies PAb421 (421) and 200.47 (HD200). The p53-cleared extracts were layered on top of a 5 to 20% (wt/vol) sucrose gradient (containing 140 mM NaCl, 10 mM Tris hydrochloride [pH 8.0], 10 mM dithiothreitol, and 1% aprotinin) with a 0.4-ml 60% sucrose pad. Protein complexes were sedimented in a Beckman SW50.1 rotor at 47,500 rpm for 3 h at 4°C. Eight 625- μ l fractions were collected from the bottom of each gradient. (The numbers 1 to 8 represent gradient fractions collected from bottom to top.) Each fraction was immunoprecipitated using the anti-T-ag monoclonal antibody PAb419, and the disrupted immune complexes were subjected to SDS-PAGE on a 10% polyacrylamide gel as described in Materials and Methods. RNA sedimentation standards (23S, 16S, and 5S rRNAs) were run in parallel gradients; these standards sedimented in fractions 2, 4, and 7, respectively. Immunoprecipitates of nonsedimented cellular extracts were included as controls in the right three lanes. The positions of T-ag (T), p73, and p53 are indicated on the right. Molecular sizes (in kilodaltons) are indicated on the left.

complexed with p53. In some experiments, a 40-kDa band was released by ATP; however, this activity was not reproducible.

To establish the degree of relationship between p73 and the heat shock 70 family of proteins, the isoelectric point and the partial proteolytic map of p73 were compared with those of known heat shock-like proteins prepared in the same manner as p73. Munro and Pelham (61) recently demonstrated that a 72-kDa protein (p72) is identical to the glucose-regulated protein GRP78 and to the immunoglobulin heavy-chain-binding protein BiP. That protein is a member of the heat shock family of proteins and is related to hsp70, having between 57 and 62% homology with the hsp70 proteins of *Drosophila melanogaster*, humans, and rats (61). However, unlike hsp70, GRP78 (BiP) is localized to the endoplasmic reticulum. It is abundant in antibody-secreting cells and can be released from its association with the immunoglobulin heavy chain by incubation with ATP. GRP78 was prepared from a mouse hybridoma cell line, m73 (27), which secretes IgG2a immunoglobulins that are directed against adenovirus E1A gene products. Immunoglobulins from cellular extracts

TABLE 1. Isoelectric points of mouse heat shock proteins isolated by using an ATP release assay^a

Heat shock protein	Reported pI ^b	Observed pI
hsp70	5.8–6.3	Not done
p73		5.2, 4.5
GRP78	5.2–5.3	4.8, 3.9

^a One-dimensional IEF was performed using p73 released from mutant T-ag (d428) and GRP78 released from immunoglobulin heavy chains by incubating immune complexes with ATP as described in Materials and Methods.

^b Reported by Pelham (64).

of m73 hybridoma cells were collected using Formalin-fixed *S. aureus*, and the precipitates were washed as described in Materials and Methods. An ATP release assay was performed, and the released material was analyzed by flat-bed IEF and SDS-PAGE.

The IEF analyses revealed a biphasic pI of 5.2 and 4.5 for the p73 released from T-ag immunoprecipitates (Table 1). This was more acidic than the pI of 5.8 to 6.3 reported for hsp70 (64). GRP78 prepared similarly (ATP-mediated release from an immunoprecipitate) also exhibited a biphasic pI (4.8 and 3.9) under the same nondenaturing IEF conditions. Again, this was more acidic than the pI (5.2 to 5.3) described for GRP78 (64). In an attempt to explain the pI data, we performed a photoaffinity analysis using [α - ^{35} S]ATP to ascertain whether p73 was binding ATP. Under the conditions of the ATP release assay, we observed binding of labeled ATP to p73, including both the p73 complexed to T-ag (pellet) and that released from it (supernatant) (data not shown). These results were consistent with the interpretation that p73 utilizes ATP for its release from T-ag. Furthermore, these results may explain the discrepant pI values exhibited by p73 and GRP78 purified by using a release assay; a more acidic pI may be displayed during IEF if ATP or ADP is bound to the protein.

Structural relatedness between p73 and GRP78 was assessed by partial proteolytic analysis. The V8 protease cleavage patterns for p73 and GRP78, purified by SDS-PAGE after the ATP release assay, were different for the two proteins (Fig. 6). This indicated that the two proteins are not very similar structurally, although they do share certain biochemical properties.

Immunoprecipitation analysis of human cells transformed by SV40 (GM847) using the anti-T-ag monoclonal antibody PAb204 resulted in the coprecipitation with WT T-ag of a protein of similar molecular weight to p73 (Fig. 7A). An ATP release assay was performed, and a p73-like component was detected in the supernatant. This protein band was excised from the gel and was compared by partial proteolytic mapping with p73 released from the d428 mutant (Fig. 7B). Comparisons of the partial digests revealed that there was substantial structural conservation between the human and rodent p73 species. These data suggest that there may be conservation of function among mammalian p73 proteins.

Identification of p73 as a member of the hsp70 family of proteins. To establish whether p73 is related to the hsp70 family of proteins, we attempted to immunoprecipitate the p73/T-ag complexes from cellular extracts of ST803 cells by using a monoclonal antibody specific for human heat shock proteins hsp72/73. A protein of approximately 73 kDa was immunoprecipitated; however, we failed to detect coimmunoprecipitation of the d428 mutant T-ag (data not shown). It is possible that coimmunoprecipitation of T-ag was not observed because the p73 in complex with T-ag may represent only a minor subpopulation of hsp70 that was not

FIG. 6. p73 complexed to T-ag is structurally different from the immunoglobulin heavy-chain-binding protein BiP (GRP78). The hybridoma cell line m73 and ST803 cells were metabolically labeled with [^{35} S]methionine and were extracted as described in the legend to Fig. 2. T-ag was immunoprecipitated with the T-ag-specific monoclonal antibody PAb419, and *S. aureus* Cowan I was added to immunoprecipitate the immunoglobulin heavy chains from the m73 hybridoma cell extracts. ATP was used to specifically release p73 from the T-ag-containing immune complexes and GRP78 from the immunoglobulin heavy chains. The released material was subjected to SDS-PAGE on a 10% polyacrylamide gel, and the labeled proteins were visualized by autoradiography. Bands corresponding to p73 and GRP78 were excised from the gel and were subjected to partial V8 proteolysis using various concentrations of V8 protease (0.5, 5.0, and 50 μ g per lane) as described in Materials and Methods. The proteolytic digests were analyzed by SDS-PAGE on 16% polyacrylamide gels, and the proteolytic fragments were visualized by fluorography.

detected among a large cellular content of uncomplexed hsp70 and hsp70-like proteins. Alternatively, the determinant recognized by the anti-hsp70 monoclonal antibody may not have been available for binding, possibly due to conformational effects on the epitope by the interaction between p73 and T-ag.

We next determined whether the anti-hsp70 monoclonal antibody could recognize p73 under other conditions. T-ag was immunoprecipitated from extracts of unlabeled ST803, ST300, and mKSA-Asc cells by PAb430 or PAb602, and the gel-separated proteins were examined by immunoblot analysis as described in Materials and Methods (Fig. 8). The anti-hsp70 monoclonal antibody N27F3-4, which did not coimmunoprecipitate T-ag from cell extracts, specifically recognized by immunoblot the p73 that was complexed to the *dl428* and WT T-ag. However, no p73-reactive protein was detected in the T-ag immunoprecipitates from ST300 cells. These results confirmed the previous data (Fig. 2A) which indicated that p73 bound to the *dl428* and WT T-ag

but not to the *dl423* mutant T-ag. Moreover, these data indicate that the anti-hsp70 monoclonal antibody could recognize p73 that had complexed to T-ag.

Partial V8 protease cleavage analyses were performed on the free 73-kDa protein immunoprecipitated from ST803 cells by using the anti-hsp70 monoclonal antibody and on the p73 released from T-ag immunoprecipitates by ATP (Fig. 9). The partial proteolytic cleavage maps indicate a high degree of structural similarity between hsp70 and p73. These results support the interpretation that p73 is a member of the hsp70 family of proteins.

Substrate specificity requirements for dissociation of p73 from T-ag. The T-ag/p73 complex is very stable. Washes of immunoprecipitates with high salt and detergent (1.0 M NaCl, 250 mM LiCl, 10 mM EDTA, and 1% SDS) did not dissociate the complex (data not shown). However, as described above, p73 could be specifically dissociated from T-ag by incubation of T-ag immunoprecipitates containing the complex in the presence of ATP. The substrate specificity for p73 was analyzed in detail. Immunoprecipitates containing the T-ag/p73 complex were prepared from ST300 cells by using PAb204, a monoclonal antibody known to inhibit the intrinsic ATPase activity of T-ag. The ATP release assay was performed, with some samples incubated with ribonucleotide triphosphates other than ATP (Fig. 10). The release of p73 was facilitated by ATP, GTP, CTP, and UTP, in decreasing order of efficiency. Nonhydrolyzable analogs of ATP and GTP were not utilized (Fig. 10 and Table 2); p73 was not released from the pellet into the supernatant in those samples. These results suggest that the p73/T-ag complex requires ATP or GTP hydrolysis for dissociation. Furthermore, the substrate utilization data suggest that all of the ribonucleotide triphosphates mediated p73 release.

It should be emphasized that the substrate specificity observed for the p73 release assay was different from that of the intrinsic ATPase activity of T-ag. ATP and dATP are the preferential substrates of the T-ag ATPase, whereas GTP, CTP, and UTP are not utilized (22). In contrast, ATP and GTP were the substrates most efficiently utilized by p73 in the release assay. CTP and UTP were utilized to a lesser extent, and dATP and ADP were not effective substrates (Table 2). These results strongly suggest that the release of p73 from T-ag immunoprecipitates is not a result of the intrinsic ATPase activity of T-ag.

DISCUSSION

The mechanisms by which SV40 T-ag mediates its multifunctional properties are not well understood, but it seems likely that T-ag must accomplish its ascribed biologic functions by interacting with components of various cellular pathways (50). Few proteins have been identified that directly interact with T-ag. The cellular protein p53 (67) and the cellular DNA polymerase alpha (20) are two recognized substrates; more recently, the product of the Rb gene has been demonstrated to associate with T-ag (16). Both p53 and DNA polymerase alpha have been shown to bind competitively to T-ag at the same carboxy-terminal site (20), whereas the binding site for the Rb gene product has been localized to an amino-terminal T-ag domain (16). These associations do not explain all of the biological characteristics of T-ag; other as yet unidentified cellular proteins must be interacting with T-ag.

The results reported in this study demonstrate that a cellular heat shock protein of approximately 73 kDa specifically associates with WT T-ag and certain mutant forms of

FIG. 7. A 73-kDa protein coimmunoprecipitated with WT T-ag from SV40-transformed human cells is structurally related to p73 complexed to mutant T-ag in mouse cells. (A) ST803 and GM847 cells were metabolically labeled with [^{35} S]methionine and were extracted as described in the legend to Fig. 2. Cell extracts were sequentially immunoprecipitated, first with the monoclonal antibody m73 (N) and then with the anti-T-ag monoclonal antibody PAb204 (204). Each bacterial pellet was incubated with ATP release assay buffer containing 1 mM ATP for 1 h at 4°C. The pellets (P) and supernatants (S) were analyzed by SDS-PAGE on a 10% polyacrylamide gel, and the labeled proteins were visualized by autoradiography. Positions of WT T-ag (T), mutant *dl428* T-ag (●), p73, and p53 are indicated on the right. (B) Bands corresponding to human (GM847) and mouse (ST 803) p73 released from T-ag immune complexes using ATP were excised from the gel and were subjected to partial proteolysis using various concentrations of V8 protease (0.5, 5.0, and 50 μ g per lane) as described in Materials and Methods.

T-ag. We found this interaction was neither a result of nonspecific binding during immunoprecipitation nor due to cross-reactivity by anti-T-ag polyclonal or monoclonal antibodies.

Because previous reports indicated that p53 itself can bind hsp70 or hsc70 (19, 30, 66, 81), one concern during this investigation was the possibility that p73 may be bound to p53 that was complexed to T-ag. However, we found that the association between T-ag and p73 was independent of p53/T-ag complex formation. Sequential immunoprecipitation analyses indicated that monoclonal antibodies directed against p53 could immunoprecipitate complexes containing p73, T-ag, and p53 and that cellular extracts cleared of p53 still contained large amounts of T-ag complexed only to p73. These results were confirmed by velocity sedimentation analysis on sucrose gradients.

The T-ag/p73 complex is very stable. Although washes of T-ag immunoprecipitates with high salt, chelating agents, and detergents failed to disassemble the complex, p73 could be dissociated from T-ag by incubation with ATP. The ATP-generated release was specific for p73 because under the same conditions p53 was not dislodged from complexes in anti-T-ag immunoprecipitates and T-ag was not released from anti-p53 immunoprecipitates. The dissociation of p73 from T-ag appears to be mediated by an ATPase activity that is intrinsic to p73. Monoclonal antibodies that inhibit the intrinsic ATPase of T-ag did not block p73 release from T-ag immunoprecipitates. In addition, the substrate specificity exhibited in the p73 release assay was different from that described for the T-ag ATPase.

Several observations indicate that p73 is related to members of the hsp70 family of proteins. p73 is nonphosphorylated and has an isoelectric point similar to that of hsp70. p73 appears to possess an ATP-dependent release activity; specific *in vitro* binding of [α - ^{35} S]ATP and [α - ^{32} P]GTP (unpublished data) to p73 could be demonstrated, and ATP hydrolysis may be necessary for release of p73 from T-ag immunoprecipitates, since nonhydrolyzable analogs of ATP did not facilitate p73 release. In this respect, p73 closely resembles hsc70, the heat shock protein that reportedly binds p53 (10, 30); hsc70 also exhibits ATP-, GTP-, and CTP-dependent activities in nucleotide triphosphate-mediated release assays but, unlike p73, does not utilize UTP. Like all members of the hsp70 family of proteins, p73 is highly conserved; partial proteolytic maps indicate that p73 bound to WT T-ag in SV40-transformed human cells was identical to p73 isolated from mutant T-ag-transformed mouse cells. In addition, p73 exhibited a partial V8 protease degradation pattern similar to that of hsp70. Finally, p73 released from T-ag was recognized by an anti-human hsp72/73 monoclonal antibody on immunoblots. It is interesting that the monoclonal antibody specific for human hsp70 was unable to immunoprecipitate the p73/T-ag complex from cellular extracts. However, on immunoblots this antibody recognized p73 complexed to T-ag as well as the released form of p73. The failure to coimmunoprecipitate the p73/T-ag complex may reflect an inability to detect a small subpopulation of hsp70 complexed to T-ag among a vast excess of uncomplexed hsp70 in the cell. Alternatively, these results might indicate that the antigenic determinant

FIG. 8. Recognition of p73 by an anti-human hsp70 monoclonal antibody on immunoblots. (A) Sequential immunoprecipitation of *d1428* from extracts of ST803 (803) cells metabolically labeled with [³⁵S]methionine, first using a control monoclonal antibody, m73 (N), and subsequently immunoprecipitated with the anti-T-ag monoclonal antibody PAb430 (430) as described in Materials and Methods. An ATP release assay was performed on each of the immunoprecipitates, and the pellets (P) and supernatants (S) from the release assay were analyzed by SDS-PAGE on 10% polyacrylamide gels. (B) Cell lines mKSA-Asc (WT), ST803 (803), and ST300 (300), expressing WT and mutant T-ag *d1428* and *d1423*, respectively, were extracted, sequentially immunoprecipitated, and assayed for p73 release as described for panel A, except that PAb602 (602) was used to immunoprecipitate the *d1423* mutant. Purified hsp72/73 from HeLa cells (provided by W. Welch) was included as a control. The proteins were transferred to nitrocellulose filters and were processed as described in Materials and Methods. The filters were probed first with the mouse anti-human hsp72/73 monoclonal antibody N27F3-4. A second antibody, rabbit anti-mouse IgG, was then added, followed by the addition of ¹²⁵I-labeled protein A. Autoradiography was performed; panel A was exposed for 20 h, and panel B represents a 1-h exposure. The positions of T-ag (T), p53, and p73 are indicated. Bands corresponding to mouse heavy (H) and light (L) chains of IgG were observed in the immunoblots of pellets of immunoprecipitated material.

recognized by the monoclonal antibody may either be hidden by the interaction with T-ag or possibly masked by some yet unidentified mechanism.

An early report (37) noted that SV40 infection of either permissive or nonpermissive cells may induce hsp70 synthesis but that T-ag itself does not bind hsp70. In contrast, our results indicate that WT T-ag is indeed capable of stable association with p73, although to a lesser extent than are certain T-ag mutants. Perhaps conformational changes introduced by the deletion of specific T-ag amino acids result in the stabilization of a normally transient complex between T-ag and p73; this may explain why certain T-ag mutants display a greater predilection for binding p73 than does WT T-ag.

FIG. 9. Relationship of p73 to mammalian heat shock protein hsp70 by partial proteolytic cleavage with V8 protease. BALB/c 3T3E cells transformed by *d1428* (ST 803) were metabolically labeled and extracted as described in the legend to Fig. 2. Cell lysates were sequentially immunoprecipitated with normal hamster serum, with anti-hsp68/70 monoclonal antibody N27F3-4 (from W. Welch), and finally, with anti-T-ag monoclonal antibody PAb419. *S. aureus* Cowan I pellets containing T-ag immune complexes were suspended and incubated with ATP as described in the legend to Fig. 4. All the samples were analyzed by SDS-PAGE on 10% polyacrylamide gels. A protein was observed in the immunoprecipitate obtained with the anti-hsp68/70 serum which comigrated with the p73 released from T-ag immune complexes. Both bands were excised and subjected to partial digestion with various concentrations of staphylococcal V8 protease (0.5, 5.0, and 50 μ g per lane). The proteolytic digests were analyzed by SDS-PAGE on 16% polyacrylamide gels, and the proteolytic fragments were visualized by fluorography. Molecular sizes (MW, in kilodaltons) are indicated on the right.

Michalovitz et al. (57) recently have shown that another in-phase deletion mutant (CTSV), which lacks the T-ag nuclear transport signal, specifically binds hsp70. They suggested that a conformational change resulting from the amino acid deletion may stabilize the interaction of hsp70 with the mutant protein. In addition, it was suggested that association of hsp70 with CTSV may influence nuclear transport of the mutant protein. However, with our panel of mutants, we have observed that the stabilization of p73 binding does not influence the transport of T-ag to the nucleus (data not shown). Several mutants that bind p73 are transported to the nucleus, and conversely, T-ag mutants that lack the amino-terminal 108 amino acids and fail to bind p73 also undergo nuclear translocation. Recent evidence also indicates that hsp70 binds to a T-ag mutant that contains an in-phase duplication in both rat and human cells (E. May, personal communication). Taken together these results indicate that certain T-ag mutations appear to stabilize the association of p73 with T-ag.

The p73-binding site has been mapped to the amino terminus of the T-ag molecule by use of the *d1425* mutant; it encodes only the amino-terminal 178 amino acids and that fragment complexed with p73. In addition, binding of p73 was not affected by internal deletions of T-ag amino acids 179 to 229, 179 to 315, or 179 to 229 plus 292 to 315. Since p73 interacts with T-ag and not with t-ag, the amino-terminal 82 amino acid residues that are in common can probably be excluded from the putative p73-binding domain of T-ag, although we have not ruled out the possibility that the interaction between p73 and T-ag involves amino acid se-

FIG. 10. Determination of nucleotide triphosphate substrate specificity for p73 release from T-ag immunoprecipitates. ST803 cells were labeled with [^{35} S]methionine and were extracted as described in the legend to Fig. 2. Cell extracts were sequentially immunoprecipitated first with the control monoclonal antibody m73 (N) and then with the anti-T-ag monoclonal antibody PAb204. Each bacterial pellet was incubated with ATP release assay buffer alone (—) or with buffer containing 1 mM of one of the following nucleotides: ATP, ATP- γ -S, GTP, GMP-PCP, GMP-PNP, CTP, or UTP. The pellets (P) and supernatants (S) were analyzed by SDS-PAGE on 10% polyacrylamide gels. The positions of T-ag (T), p73, and p53 are indicated on the right. ●, Position of the 68-kDa cellular protein that cross-reacted with PAb204.

quences surrounding the first and second exon junctional region. Truncated forms of T-ag that lacked the first 108 amino acids did not bind p73. From these data, the p73-binding domain on T-ag can be provisionally delimited to the amino acid residues between 82 and 178. This putative p73-binding region can be further divided into two separate regions (82 to 109 and 153 to 178), because hsp70 was reported to associate with the CTSV mutant which deletes amino acids 110 to 152 (57). Interestingly, the binding

TABLE 2. Substrate specificity for the release of p73 from T-ag immunoprecipitates in a release assay^a

Substrate	p73 release ^b
ATP	++++
GTP	+++
CTP	++
UTP	++
dATP	+
ADP	±
ATP- γ -S	± to —
AMP-PNP	—
GMP-PNP	—
GMP-PCP	—

^a An ATP release assay was performed on T-ag immunoprecipitates from ST803 cells. ST803 cells were labeled with [^{35} S]methionine and were extracted as described in the legend to Fig. 2. Cell extracts were sequentially immunoprecipitated first with the control monoclonal antibody m73 and then with the anti-T-ag monoclonal antibody PAb204. Each bacterial pellet was incubated with ATP release assay buffer alone or with buffer containing one of the indicated nucleotides (1 mM). The pellets and supernatants were analyzed by SDS-PAGE on 10% polyacrylamide gels. Proteins were visualized by autoradiography.

^b Symbols: ++++ to —, arbitrary scale indicating the relative amount of radiolabeled p73 released into the supernatant fluid in the presence of a given substrate in the release assay (++++, most release; —, no detectable release).

domain for the Rb gene product has been identified within this same region of T-ag. It will be informative to determine if there is any competition for binding sites between these two proteins. The interaction between T-ag (an oncogene) and the Rb gene product (an anti-oncogene) has important implications for the study of oncogenesis and regulation of growth control.

Pelham (64) has proposed that hsp70 may interact with overexpressed or inappropriately folded protein molecules. This phenomenon has been noted in cells expressing a mutant oncoprotein, such as p53 (19, 81) or polyomavirus middle T-ag (84). On the basis of such observations of heat shock proteins binding abnormal proteins and thereby possibly targeting them for degradation or transport out of the cell or both, heat shock proteins have been postulated to function as mediators of intracellular transport (64). However, the p73/T-ag complex is relatively long-lived (unpublished data), suggesting that p73 is not serving to accelerate the degradation of mutant T-ag.

Numerous properties have been ascribed to hsp70 and hsp70-like molecules, including conference of thermotolerance (51, 60), uncoating ATPase of clathrin-coated pits (7, 83), autophagy (59), polyadenylate binding (75), fatty acid binding (24), association with a surface glycoprotein (31), and association with cytoskeletal elements, such as microtubules (53, 85). In addition, hsp70 may play a role during normal development and growth (14). Expression of hsp70 or its cognate genes has been reported to be cell cycle dependent (58) and developmentally regulated during sporulation in yeast (41), oogenesis in *D. melanogaster* (41), and spermatogenesis in mice (90), as well as during erythroleukemic cell differentiation (29).

The biologic significance of p73/T-ag complex formation is yet to be determined, but several possible p73 function(s) can be envisioned. p73 may interact with T-ag during the formation of replication complexes and may participate in SV40 DNA replication. T-ag itself is essential for viral replication, functioning as an origin-specific DNA-binding protein (67) with helicase activity (40, 86) and associating with the cellular DNA polymerase α (20). The procaryotic initiation complexes of bacteriophage lambda consist of an origin-specific DNA-binding protein (λ O), a helicase (*dnaB*), a protein that interacts with the origin-binding protein and helicase (λ P), and two heat shock proteins (DnaK and DnaJ), one of which is similar to hsp70 (52). The eucaryotic hsp70 and the *E. coli dnaK* gene product share evolutionary homology (50% at the sequence level) and conservation of enzymatic function with respect to p53-binding and ATP-mediated release (1, 10). Additional interesting similarities are that the *dnaK* gene product is essential for *E. coli* and lambda phage replication (92) and is reported to interact with the nuclear oncoprotein p53 (10).

Another possible function for p73 may be that of a molecular chaperone (a mediator of intracellular protein-protein interactions). Examples of proteins with similar properties are found in procaryotic systems (28). Evolutionary conservation of these proteins is exhibited by the identification of similar proteins in mitochondria from yeast, frog, plant, and human cells (56). The hsp70 family of proteins may have evolved as an analogous intracellular transport system in eucaryotes serving as ATP-dependent "unfoldases" (8, 69). Consistent with this hypothesis, several recent reports have indicated that the 70-kDa heat shock proteins are important in mediating protein translocation across membranes (8, 17).

Finally, T-ag is unique among viral oncogenes because of

its dual location in both the nucleus and plasma membrane (6). Very little is known about how T-ag is transported to the cell surface; recent studies have ruled out utilization of the secretory pathway (33, 77). As T-ag apparently does not migrate through conventional pathways to the cell surface, some unidentified mechanism may be involved. Because hsp70-like proteins have been identified as mediators of intracellular transport, the association of p73 with T-ag may reflect some transport-related process.

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