Immunological Evidence for the Association of p53 with a Heat Shock Protein, hsc70, in p53-plus-*ras*-Transformed Cell Lines

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A rabbit antiserum was prepared against the C-terminal peptide of 21 amino acids from the human heat shock protein hsp70. These antibodies were shown to be specific for this highly inducible heat shock protein (72 kilodaltons [kDa] in rat cells), and for a moderately inducible, constitutively expressed heat shock protein, hsc70 (74 kDa). In six independently derived rat cell lines transformed by a murine cDNA-genomic hybrid clone of p53 plus an activated Ha-*ras* gene, elevated levels of p53 were detected by immunoprecipitation by using murine-specific anti-p53 monoclonal antibodies. In all cases, the hsc70, but not the hsp70, protein was coimmunoprecipitated with the murine p53 protein. Similarly, antiserum to heat shock protein coimmunoprecipitated p53. Western blot (immunoblot) analysis demonstrated that the hsc70 and p53 proteins did not share detectable antigenic epitopes. The results provide clear immunological evidence for the specific association of a single heat shock protein, hsc70, with p53 in p53-plus-*ras*-transformed cell lines. A p53 cDNA clone, p11-4, failed to produce clonable cell lines from foci of primary rat cells transfected with p11-4 plus Ha-*ras*. A mutant p53 cDNA clone derived from p11-4, SVKH215, yielded a 2- to 35-fold increase in the number of foci produced after transfection of rat cells with SVKH215 plus Ha-*ras*. When cloned, 87.5% of these foci produced transformed cell lines. SVKH215 encodes a mutant p53 protein that binds preferentially to the heat shock proteins of 70 kDa compared with binding by the parental p11-4 p53 gene product. These data suggest that the p53-hsc70 protein complex could have functional significance in these transformed cells.

The cellular tumor antigen p53 has been found at elevated levels in some human tumors and in many diverse transformed cell lines, including those expressing the simian virus 40 (SV40) large T antigen (T antigen) (2–4, 14, 23). The elevated levels of p53 found in T antigen-transformed cell lines result from the formation of an oligomeric protein complex between T antigen and p53 which appears to increase the stability of p53 protein (14, 18). Recent results suggest that p53 may be essential for large T antigen-mediated transformation (16) and that transformation-negative T antigen mutants can be suppressed by overexpression of p53 (15). Transfection of the gene encoding p53 can lead to immortalization of primary cells in culture (10), and cotransfection with an activated *ras* oncogene results in morphological transformation of the recipient cells (5, 19). p53 is both abundant and stable in such cells (22), as it is in many other transformed cell lines (17, 18), despite the lack of the SV40 T antigen. One way to explain the long half-life (stability) of p53 in these cells is to assume that a cellular protein analogous to T antigen interacts with and stabilizes p53. Several different cell lines expressing a stabilized p53 protein in the absence of T antigen have been shown to contain p53 complexed to a 68-kilodalton (kDa) protein (termed p68) (7, 21, 26). COS7 cells transiently expressing certain mutant p53 proteins also contain a p68-p53 complex which coimmunoprecipitates when p53-specific monoclonal antibodies are used (27). It has been suggested that p68 in one transformed rat cell line is a heat shock protein of the 70-kDa (hsp70) family (22). The assertion that p68 is identical to one or more members of the hsp70 family of proteins is based upon comigration of the p68 and hsp70 proteins in one- and two-dimensional gel electrophoresis analysis of cell extracts from a p53-plus-*ras*-transformed cell line (clone 6) (22).

The heat shock proteins are a ubiquitous group of proteins which are often present at low levels in normal cells. Upon physiological stress, the synthesis of these proteins dramatically increases and the pattern of total cellular protein synthesis is characteristically altered (9, 29, 30). In human cells, the hsp70 family of proteins is composed of at least three closely related proteins (>80% homology at the amino acid level) which have been termed hsp70, hsc70, and hsc70 (20). hsp70 is highly heat inducible and hardly detectable under normal conditions. hsc70 is also quite heat inducible but has a high basal level which is regulated in a cell-cycle-dependent fashion (12, 33). This protein may not have a counterpart in rodent cells (20). hsc70 is only slightly heat inducible and is found at higher levels in growing cells than in resting cells (20). The present communication provides proof, based upon immunological criteria, that p68, the protein complexed with p53, is a member of the hsp70 family. Antiserum directed against C-terminal epitopes representing the last 21 amino acids of these proteins is specific for at least the hsp70 and hsc70 proteins. These antibodies detected the hsc70 protein (but not the hsp70 protein) in a complex with p53 in several independently derived p53-plus-*ras*-transformed cell lines. A mutant p53 protein that more efficiently binds with hsc70 proteins appears to transform rat cells with the *ras* oncogene more efficiently than does the parent p53. These data suggest that the p53-hsc70 complex may have some functional significance.

MATERIALS AND METHODS

Production of the synthetic peptide. The peptide NH₂-CGPGGFGAQQGPKGGSGSGPTIE-COOH, representing 21 amino acids of the carboxyl-terminal sequence of a member of the human 70-kDa heat shock protein (hsp70) family (9) (plus a cysteine residue added to the amino terminus of this peptide), was prepared on an Applied Biosystems model 403A peptide synthesizer. The average step yield was

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99.46%, resulting in 97.8% of the theoretical maximum yield. After hydrogen fluoride cleavage, extraction with cold anhydrous ether, and lyophilization, the peptide was recovered and subjected to reverse-phase high-performance liquid chromatography, amino acid analysis, and sequential Edman degradation. A single peptide peak detected at 216 nm was identified by using two separate chromatographic conditions. Amino acid analysis and amino-terminal sequencing revealed that this peptide was homogeneous in chemical composition and sequence.

Immunization. Five milligrams of commercial keyhole limpet hemocyanin (Calbiochem-Behring) and 25 mg of peptide were dissolved in 5 ml of water at pH 5.0. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was added to yield a concentration of 0.1 M, and pH 5 was maintained. After being stirred for 18 h at 4°C, the conjugated peptide solution was dialyzed against 25 mM (NH₄)HCO₃–10 mM glycine. After lyophilization, the conjugated peptide concentration was adjusted to 5 mg/ml.

Male New Zealand White rabbits were inoculated intradermally with 400 µg of keyhole limpet hemocyanin-conjugated peptide in 1 ml of 50% Freund complete adjuvant–50% phosphate-buffered saline (PBS). Three weeks after the initial injection, the rabbits were boosted with 40 µg of conjugated peptide in 1 ml of 50% Freund incomplete adjuvant–50% PBS. Boosts were repeated every 10 days for a total of 2 months. After the fourth boost 30 ml of blood was collected from each rabbit, and the serum was cleared by centrifugation. The rabbits were bled two additional times at 2- to 3-week intervals prior to exsanguination, which occurred 13 weeks after the initial immunization.

Transformation of primary REF by p53 and activated Ha-ras. Primary rat embryo fibroblasts (REF) were prepared from 14- to 15-day-old whole rat embryos or from the back skins of 19-day-old Fischer 344 rat embryos. A total of 2.8×10^5 secondary or tertiary REF were seeded per 60-mm dish and 24 h later were cotransfected by the calcium phosphate procedure (32) with 1.25 µg of plasmid pT24 encoding activated Ha-ras (6), 1.25 µg of the p53-encoding test plasmid, and 10 µg of salmon sperm DNA as carrier. The p53 clones tested were pLTRp53cG (a murine cDNA-genomic hybrid under the control of the Harvey murine sarcoma virus long terminal repeat), which was a kind gift from M. Oren (11); p11-4 (a murine cDNA under the control of the SV40 enhancer/promoter) (27); and SVKH215 (a derivative of p11-4 containing a linker-insertion mutation at amino acid 215) (27). The cells were transfected for 17 to 18 h, refed with Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FCS), and allowed to recover for an additional day prior to being passaged at a 1:3 dilution. Cells were refed at 4- to 5-day intervals. Morphologically distinct foci were visible 7 to 10 days after subcultivation. At 12 to 15 days after subcultivation, selected foci were cloned and expanded as cell lines. The remaining cells were stained with crystal violet, and foci were counted.

Cell lines, labeling, and immunoprecipitation. All cell lines were maintained in DMEM–10% FCS and incubated at 37°C in a humidified 5% CO₂ atmosphere. Cells were heat shocked by replacing the medium with medium prewarmed to 41 or 43°C and by continuing incubation at the heat shock temperature for either 30 min or 2 h. The cells were prepared for labeling by two washes in warm PBS followed by the addition of methionine-free DMEM–2% FCS containing 25 to 50 µCi of [³⁵S]methionine per ml. Incubation in this labeling medium was continued for 30 min at 37°C.

At the end of the labeling period, cells were washed twice

with cold PBS, scraped from the plate, pelleted at 4°C, and frozen at –80°C. For immunoprecipitation, pellets were quickly thawed and lysed for 20 min at 4°C in 1 ml of IP lysis buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride [from freshly prepared 100 mM stock in ethyl alcohol]) for 20 min. Lysates were then sonicated for 10 s at setting 5 on a Branson sonicator, and the lysate was cleared by centrifugation. Lysates were preadsorbed with 50 µl of a 10% suspension of fixed staphylococcal protein A. Equivalent amounts of trichloroacetic acid-insoluble, radioactively labeled proteins were immunoprecipitated by the addition of nonimmune rabbit serum, antiserum to heat shock protein peptide, or the anti-p53 monoclonal antibody RA3-2C2 (25) or PAb421 (8). After 30 min on ice, 50 µl of protein A suspension was added, and the mixture was allowed to react for an additional 30 min. Immunocomplexes were collected by centrifugation and washed three times with SNTE (5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris [pH 7.4], 5 mM EDTA) at 4°C and once with RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% [wt/vol] sodium deoxycholate) at 4°C. The pellet was then suspended in SDS-polyacrylamide gel electrophoresis sample buffer (125 mM Tris [pH 6.8], 4% SDS, 10% glycerol, 0.005% bromophenol blue, 10% 2-mercaptoethanol) and boiled for 5 min. Samples were loaded on a 5% polyacrylamide stacking gel and resolved on 7.5% polyacrylamide. Gels were fluorographed in 22% PPO (2,5-diphenyloxazole) in dimethyl sulfoxide, dried, and exposed to Kodak XAR-5 film at –80°C.

Unlabeled proteins separated similarly on polyacrylamide gels were transferred to nitrocellulose at 200 mA for 6 to 18 h. Filters were blocked overnight at 4°C in 5% bovine serum albumin (pH 7.4) (blocking buffer) and then incubated with either PAb421 diluted 1:1 in blocking buffer or antiserum to hsp70 diluted 1:200 in 50% blocking buffer for 60 min at 37°C. Filters were washed with PBS–300 mM NaCl and subsequently reacted with 1 µCi of ¹²⁵I-labeled protein A in blocking buffer for 60 min at room temperature. Excess label was washed from the filters with three changes of PBS–300 mM NaCl; subsequently, the filters were dried and exposed to XAR-5 film at –80°C.

RESULTS

Characterization of antiserum to heat shock protein peptide. Serum obtained from rabbits immunized with the keyhole limpet hemocyanin-synthetic peptide CGPGGFGA-QGPKGGSGSGPTIE, derived from the C terminus of a 70-kDa heat shock protein family member, hsp70 (9), was shown to bind to polyvinyl chloride microtiter plates coated with lysozyme-peptide conjugate to a serum dilution of 1:100,000. Wells coated with free peptide bound the serum to a dilution of 1:25,000, and no response was seen to either free lysozyme or bovine serum albumin; this observation suggests specificity of the serum for the peptide (data not shown). To determine the specificity of the serum for the parent protein, extracts of HeLa cells maintained at 37 or 41°C were immunoprecipitated with antiserum to the hsp70 peptide. The serum specifically precipitated a 71-kDa (hsc70) and a 73-kDa (hsp70) protein from HeLa cells maintained at 37°C (Fig. 1a, lane B). Upon heat shock at 41°C, both immunoprecipitated proteins increased significantly in abundance; the 71-kDa species showed the greatest increase in expression (Fig. 1a, lane D).

The serum also recognized proteins in REF, as shown in Fig. 1b. Here the response was slightly different from that in

HeLa cells. In REF maintained at 37°C, the serum specifically immunoprecipitated a single protein of 74 kDa (hsc70) (Fig. 1b, lane B), the increase in abundance of which at 41°C was accompanied by the appearance of a 72-kDa species (hsp70) (Fig. 1b, lane D). An ~60-kDa band was immunoprecipitated repeatedly by some nonimmune rabbit sera (Fig. 1b, lanes A and C), including commercial rabbit immunoglobulin G (IgG) (data not shown). Other batches of nonimmune serum and the immune serum used in this study failed to immunoprecipitate this band, suggesting that the protein is not antigenically related to hsp70. Thus, antiserum to the synthetic heat shock peptide specifically immunoprecipitated at least two 70-kDa proteins which display a typical heat shock response (29, 30). Furthermore, this immunoprecipitation was totally inhibitable in both HeLa and REF cells by preincubation of the serum with 100 pmol of heat shock protein peptide, whereas incubation with an equivalent concentration of a nonspecific (not sequence-related) peptide had no effect (data not shown). Antiserum to heat shock protein peptide showed good specificity in recognizing hsc70 and hsp70 proteins.

p53 and hsp70 coimmunoprecipitate in p53-plus-*ras*-transformed cell lines. Immunoprecipitation of p53 from the p53-plus-*ras*-transformed REF cell line clone 6 (22), using a monoclonal antibody directed against p53, resulted in the coimmunoprecipitation of p53 and a 70-kDa protein (p68), which comigrated with rat hsp70 (22). To ascertain the identity of the 70-kDa protein, [³⁵S]methionine-labeled ex-

FIG. 1. Immunoprecipitation of heat shock proteins with rabbit antiserum. (a) Plates (10-cm diameter) of subconfluent HeLa cells were maintained at 37 or 41°C for 2 h. The cells were labeled with [³⁵S]methionine, and equivalent amounts (10⁶ cpm) of extract were immunoprecipitated with nonimmune rabbit serum or anti-heat shock peptide serum as described in Materials and Methods. Immunoprecipitates were boiled for 5 min in the presence of 10% 2-mercaptoethanol and 2.0% SDS and run on 7.5% polyacrylamide at 50 V overnight. (b) Secondary REF cells (4.7 × 10⁶) were maintained at 37 or 41°C for 2 h and labeled at 37°C as described for panel a. Immunoprecipitation was from 6.75 × 10⁵ cpm. Lanes: M, molecular weight markers (in kilodaltons); A and C, control nonimmune rabbit serum; B and D, antiserum to heat shock protein peptide.

FIG. 2. Coimmunoprecipitation of p53 and heat shock proteins in clone 6 cells. Dishes (60-mm diameter) containing approximately 10⁶ clone 6 cells were maintained at 37 or 41°C for either 30 min or 2 h. After heat shock, all plates were labeled for 30 min at 37°C with 50 μ Ci of [³⁵S]methionine in 1 ml of methionine-free DMEM-2% FCS. Pellets were collected and extracted, and equivalent amounts of trichloroacetic acid-insoluble radioactivity (5 × 10⁵ cpm) were used for immunoprecipitation and were run on a polyacrylamide gel as described in Materials and Methods. Lanes: M, molecular weight markers; m, extract of SV40 T antigen-transformed cell line SVT2 immunoprecipitated with PAb421 (the arrow indicates murine p53; the double arrowhead indicates T antigen); A, D, and G, control nonimmune rabbit serum; B, E, and H, anti-p53 PAb421; C, F, and I, antiserum to heat shock protein peptide.

tracts of clone 6 cells maintained either at 37°C or under heat shock conditions were subjected to immunoprecipitation with the p53-specific monoclonal antibody PAb421 (8) or with antiserum to hsp70 peptide (Fig. 2). PAb421 precipitated a 53-kDa protein (Fig. 2, lane B) which comigrated with authentic murine p53 from the SV40-transformed cell line SVT2 (Fig. 2, lane m). As was previously observed (22), PAb421 coimmunoprecipitated a protein of 74 kDa, hsc70 (Fig. 2, lane B). At 37°C, antiserum to heat shock protein peptide precipitated hsc70 (74 kDa), as well as a 53-kDa protein comigrating with p53 (Fig. 2, lane C). Under heat shock conditions, antiserum to heat shock protein peptide precipitated increased amounts of hsp70 (72 kDa) (Fig. 2, lanes F and I), whereas anti-p53 antibody continued to coimmunoprecipitate only the single hsc70 (74 kDa) species (Fig. 2, lanes E and H) from these extracts.

To determine whether this association of p53 and hsc70 was specific for the clone 6 cell line or whether it was a more general result of transformation by p53, five independent cell lines transformed by the p53 clone pLTRp53cG (11) and pT24-encoding H-*ras* (6) were assayed for coimmunoprecipitation of p53 and hsc70. High levels of murine p53 protein recognized by a murine-specific monoclonal antibody (RA3-2C2) (25) were being produced in all five cell lines (Fig. 3, clones A1 to A4 and B1 [lanes C]). p53 levels in these transformed cells were much higher than those detected in

FIG. 3. Coimmunoprecipitation of p53 and hsp70 from five independent p53-plus-*ras*-transformed cell lines. Normal secondary REF and five cell lines transformed by pLTRp53cG plus Ha-*ras* (maintained at 37°C) were labeled and collected as described in Materials and Methods. At time of labeling, cell density on a 10-cm plate was as follows: A1, 6.5×10^6 ; A2, 2.5×10^6 ; A3, 4.8×10^6 ; A4, 6.3×10^6 ; B1, 1.3×10^6 ; REF, 4.7×10^6 . Immunoprecipitations were from 6.75×10^5 cpm. The arrow indicates migration of murine p53 from SVT2 cells. Molecular weight markers (in kilodaltons) are indicated to the left of the gel. Lanes: A, control nonimmune rabbit serum; B, anti-p53 PAb421; C, anti-mouse p53 monoclonal antibody RA3-2C2; D, antiserum to heat shock protein peptide.

normal REF cells (Fig. 3, REF [lanes B and C]). In each of these transformed cell lines, the hsp70 protein was precipitated when anti-p53 antibodies were used (lanes B and C). This protein comigrated with the hsp70 that was immunoprecipitated by polyclonal antiserum to hsp70 peptide (lanes D). Further, this antiserum to hsp70 precipitated a 53-kDa protein in each of the five transformed cell lines. Thus, expression of p53 in many independently derived transformed rat cell lines results in the ability to coimmunoprecipitate p53 and hsp70.

A reciprocal immunoblot of clone 6 extracts indicates that

p53 and hsc70 are physically associated. Coimmunoprecipitation of two proteins can be due to either shared epitopes or the physical association of the proteins. To demonstrate that p53 and hsc70 are associated in clone 6 extracts, unlabeled extracts, prepared from cells either maintained at 37°C or heat shocked, were subjected to immunoprecipitation with either PAb421 or antiserum to hsp70. The denatured (by SDS treatment), separated proteins were transferred to nitrocellulose paper and then Western blotted with either PAb421 (Fig. 4a) or antiserum to hsp70 (Fig. 4b). Antiserum to heat shock protein coimmunoprecipitated a 53-kDa pro-

FIG. 4. Reciprocal immunoblot of proteins immunoprecipitated by anti-p53 and anti-heat shock protein antibodies. Plates (10-cm diameter) of 5.6×10^6 clone 6 cells were maintained at 37 or 43°C for 30 min. After heat shock, cell extracts were prepared as described in Materials and Methods. A 200- μ g amount of total protein was used for immunoprecipitation by nonimmune rabbit serum (lanes A and D), anti-p53 PAb421 (lanes B and E), or antiserum to heat shock protein (lanes C and F). Immunocomplexes were boiled in 30 μ l of SDS sample buffer, loaded on two equivalent sets of wells on a single 7.5% polyacrylamide gel, and run overnight at 50 V. Separated proteins were transferred to nitrocellulose as described in Materials and Methods to generate two identical filters. Filters were blotted with PAb421 diluted 1:1 in blocking buffer (a) or with antiserum to heat shock protein peptide diluted 1:200 in 2.5% bovine serum albumin (b) and then were reacted with 1 μ Ci of 125 I-labeled protein A. After being washed, filters were exposed to XAR-5 film at -80°C for 70 h. m, Extract from SVT2. Molecular weight markers (in kilodaltons) are indicated to the left of each panel. The arrow in panel a indicates murine p53. The arrows in panel b indicate rabbit IgG (see text).

TABLE 1. Frequency of transformation by p53 wild-type and mutant plasmids

Plasmid ^a	No. of foci/10 ⁶ cells (avg) ^b	% (no.) of cloned foci ^c
<i>ras</i> + p11-4	6, 9, 1, 1 (4.25)	0 (0/9)
<i>ras</i> + SVKH215	16, 18, 7, 35 (19.0)	87.5 (14/16)

^a The activated Ha-*ras* gene in plasmid pT24 plus the wild-type p53 cDNA (p11-4 [27]) or the mutant p53 cDNA SVKH215, both under SV40 enhancer-promoter control.

^b Results from four independent experiments are listed. Foci produced by p11-4 tended to be smaller and showed less aberrations from normal morphology than those produced by SVKH215.

^c Clonability numbers were determined for cDNAs under the Harvey murine sarcoma virus promoter. All p11-4-plus-*ras* foci reverted to normal morphology and failed to produce cell lines upon cloning. Most KH215-plus-*ras* foci cloned well to produce cell lines with an altered morphology.

tein that was recognized by PAb421 (Fig. 4a, lanes C and F), and PAb421 coimmunoprecipitated a 74-kDa protein, hsc70, which was recognized by antiserum to hsp70 peptide (Fig. 4a, lanes B and E). This result demonstrates that only hsc70 is associated with p53. Although hsp70 is present in the extract, it is not detectable when PAb421 is used to immunoprecipitate p53 and to coimmunoprecipitate the hsc70 protein. The bands at ~55 kDa in Fig. 4b are the result of protein A binding to rabbit IgG. That these bands are distinct from p53 is suggested by the following points. (i) The protein migrates at a higher molecular mass than does p53 (Fig. 4b, arrows). (ii) The band is distinct in appearance from p53 (compare Fig. 4a, lane C, with Fig. 4b, lane C). (iii) The 55-kDa band is absent from lanes immunoprecipitated with the (mouse-derived) monoclonal antibody PAb421 (Fig. 4b, lanes B and E), although these immunocomplexes should contain the highest levels of p53 (Fig. 4a, lanes B and E). p53 from SVT2 is also not detected by antiserum to hsp70 (Fig. 4b, lane m). In addition, denatured immunoglobulin heavy chains can be detected by Western blotting using ¹²⁵I-labeled protein A directly, without the addition of second antibody (data not shown). Furthermore, p53 can be coimmunoprecipitated by using affinity-purified anti-hsp70 antiserum, and Western blotting of the separated proteins with anti-hsp70 antiserum reveals only the 70-kDa doublet (data not shown). This is because there is a lower concentration of heavy chains in the immunoprecipitate. The absence of a detectable p53 band on the filter blotted with anti-hsp70 peptide antiserum and the corresponding absence of hsp70/hsc70 on the autoradiogram when PAb421 was used in the immunoblot proves that these proteins contain distinct epitopes and that these antisera do not cross-react with these proteins. These data suggest that p53 and hsc70 are physically associated in cell extracts derived from p53-plus-*ras*-transformed cells.

The p53 mutant protein that preferentially binds to the heat shock proteins has an enhanced transforming ability. The preceding experiments demonstrate that some of the p53 protein, present in a p53-plus-*ras*-transformed cell line, is complexed with the hsc70 protein. Although six of the six independently derived cell lines examined for this p53-hsc70 complex contained it, the significance of such protein interactions remains unclear. Mutant p53 cDNA clones have been obtained (27) which produce an altered p53 protein that fails to bind to the SV40 large T antigen and preferentially binds with a cellular protein, p68 (27), now identified as the heat shock protein (unpublished results). This mutant, KH215, binds to the heat shock protein under conditions in which the parent cDNA clone (wild type) does not interact

with p68 (27). If production of a p53-hsc70 protein complex plays a role in the transformation process, then KH215 might enhance the transformation frequency. To test these ideas, secondary REF were transfected with p11-4 (wild-type p53 cDNA clone [27]) and an activated *ras* gene or with KH215 (an insertion mutant derived from p11-4) and an activated *ras* gene. In several independent experiments, selected foci were cloned and the cells derived from them were passaged to determine whether a reproducible cloned cell line with a transformed phenotype could be obtained. The results of these experiments are presented in Table 1. In four independent experiments, the *ras*-plus-KH215-transfected cells produced more foci than the cells transfected with p11-4 plus *ras*. KH215 plasmids were 4.5-fold more effective on the average (with a range of 2- to 35-fold more effectiveness) than p11-4 plasmids at producing transformed foci with *ras*. More convincing was the fact that none of the p11-4-plus-*ras* foci could produce an established cell line. Upon cloning and subsequent passage, the cells appeared to be normal. With sequential passage, the cultures senesced. Most KH215-plus-*ras*-derived foci (87.5%) produced cell lines with a transformed phenotype (small rounded cells which formed grapelike clusters which sloughed off into the medium). Some of these cell lines analyzed synthesized high levels of mouse p53 complexed with heat shock proteins (data not shown).

DISCUSSION

A specific antiserum directed against the mammalian 70-kDa heat shock proteins (hsp70 family) was produced, characterized, and used to immunoprecipitate these proteins from extracts of cells transformed by p53 plus *ras*. It was demonstrated that either anti-p53 antibody or antiserum against hsp70 peptide is capable of precipitating both a 53-kDa protein and an hsc70 protein from several REF cell lines transformed with p53 and activated Ha-*ras*. Western blot analysis indicates that these two proteins are indeed p53 and the higher-molecular-weight member of the rat hsp70 doublet (hsc70) (20). The results presented in the present communication strongly argue that the p53-hsc70 protein complex is a specific one and not the result of an artifactual binding of the hsp70 family of proteins to p53 (and other proteins) in cell extracts. There are several reasons for this theory. First, p53 has been shown to associate specifically with hsc70, not hsp70, even when hsp70 is present at higher levels (of labeled protein) in these extracts. hsc70 and hsp70 share greater than 80% amino acid homology, suggesting that the binding of only one of these proteins is a meaningful or functional association. Second, the p53-hsc70 complex is resistant to high salt concentration (0.5 M NaCl) and strong detergents (1% Triton X-100, 0.1% SDS, and 1% deoxycholate) in the washing buffers. These conditions are considered stringent. Third, the association of hsc70 and p53 could be demonstrated in many independently derived cell lines transformed by p53 plus *ras*, showing a more general and presumably significant impact of this complex. In all of these cell lines, murine p53 proteins were expressed at high levels.

Heat treatment of clone 6 cells results in the production of hsp70 (72 kDa) and hsc70 (74 kDa), both of which are recognized by the antisera to C-terminal peptide. The hsc70 protein is only slightly inducible by heat shock, whereas the hsp70 protein is greatly inducible by heat shock (Fig. 1b and reference 20). The results presented in the present communication (Fig. 2 to 4) demonstrate that p53 is associated preferentially or specifically with the hsc70 species of pro-

tein, not the hsp70 protein, even when the latter protein is in abundance in the cell extract. Although this finding is in contrast to the results of Pinhasi-Kimhi and colleagues (22), who used this same clone 6 cell line, the conditions of these experiments were different. They found multiple heat shock proteins (in the hsp70 family) associated with p53 derived from clone 6 cells. In the experiments presented in the present paper, the RIPA buffer washing procedure used for immunoprecipitation is a more stringent criterion for coimmunoprecipitation than that used by Pinhasi-Kimhi et al. (22). It remains possible that the nature of the associations and binding constants between hsp70-p53 and hsc70-p53 are different and that more-stringent washing procedures selectively dissociate weaker complexes.

The significance of a p53-hsc70 complex in p53-plus-*ras*-transformed cells is at present unclear. Two lines of evidence suggest that this association is not trivial or fortuitous. First, a good correlation exists between high levels of p53 and the presence of p53-hsc70 complexes in several independently derived p53-plus-*ras*-transformed cell lines. Six of six cell lines selected for the transformed phenotype have the p53-hsc70 complex. Second, a mutant p53 protein that binds preferentially to hsc70 (called p68 in reference 27) is encoded by a cDNA clone (KH215) which has a much-enhanced frequency of transformation of rat cells with *ras* when compared with the parental cDNA clone (p11-4). In these experiments, the p53 cDNA clone, p11-4, produced transient foci that failed to be propagated upon cloning and passage. KH215, which is a linker-insertion mutant derived from p11-4, efficiently produces foci of transformed cells which give rise to stable cell lines. Hybrid cDNA-genomic clones of wild-type p53 (pLTRp53cG [11]) produce foci with *ras* that are readily clonable and that form transformed cell lines. Why the p11-4 cDNA only produces a transient focus that fails to clone or propagate is presently under investigation. KH215, which synthesizes a protein with an altered conformation (fails to bind SV40 large T antigen and PAb246 [27]), corrects this defect or behaves more like a form of p53 that is responsible for transformation.

Finally, it is interesting that hsc70, like p53, is found in higher concentrations in growing cells than in resting cells (20, 24). Similar coordinate regulation suggests that these proteins could be involved in similar processes related to control of cell growth or division. The human hsc70 protein has been reported to be homologous to the bovine brain uncoating ATPase which is required for the breakdown of clathrin baskets upon hydrolysis of ATP (1, 13, 28, 30). hsc70, which moves to the nucleus under heat shock conditions, may be responsible for the recognition of structurally altered proteins (29, 31). Perhaps overexpression of p53 or structural alterations of p53 mediate the interaction of hsc70 and p53, which is a functionally normal response to an altered oncogene product. Alternatively, hsc70-p53 complexes could result in the activation of a pathway that leads to the transformed phenotype.

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