Heat Shock Protein 84 Forms a Complex with Mutant p53 Protein Predominantly within a Cytoplasmic Compartment of the Cell*

Bahman Sepehrnia, I. Benjamin Paz, Gargi Dasgupta, and J amil Momand†

From the Department of Cell and Tumor Biology, City of Hope National Medical Center, Duarte California 91010-0269

The p53 tumor suppressor protein plays a critical role in inhibiting cellular proliferation, suppressing gene amplification, and preventing oncogene-mediated cellular transformation (1–3). In response to DNA damage, p53 binds specific DNA sequences and increases the transcription of certain genes (for reviews, see Refs. 4 and 5). Some of these p53-regulated gene products, such as p21Cip-1 and Gadd45, can inhibit cell cycle progression and accumulation of the p53 tumor suppressor protein within the nucleus which leads to cell cycle arrest or apoptosis. In some cases, however, wild-type p53 and some mutant forms of p53 reside in the cytoplasm of cancer cells. To understand the mechanism responsible for its cytoplasmic retention, studies were undertaken to determine if unique proteins form a complex with mutant p53 within the cytoplasm of transformed cells. One protein, with an apparent molecular mass of 92 kDa (p92), was observed to form a complex with a temperature-sensitive mutant p53 (TSp53Val-135) in the cytoplasm of transformed rat embryo fibroblasts at the non-permissive temperature. p92 copurified with TSp53Val-135 on a p53-specific immunofinity column and a gel filtration column. The protein was purified to homogeneity and identified as hsp84 by partial amino acid sequence analysis. hsp84 is a member of the hsp90 class of proteins. At the non-permissive temperature, TSp53Val-135 and hsp84 co-localized in the cytoplasm near the nuclear envelope. At the permissive temperature, TSp53Val-135 resides in the nucleus and expresses a “wild-type like” conformation. Under these conditions hsp84 continued to reside in the cytoplasm and little or no hsp84 formed a complex with p53. The results suggest that hsp84 binds mutant p53 in a spatial and/or conformation dependent manner.

Upon DNA backbone strand cleavage, the p53 protein level increases three to 5-fold and accumulates within the nucleus (16–18). Mutations resulting in loss-of-function are often observed in p53 genes derived from human cancers (19). In some breast cancers and neuroblastomas, however, high levels of wild-type p53 are observed in the cytoplasm (20, 21). It has been hypothesized that the inability of p53 to reside in the nucleus in these cancers may result from a second-site mutation that leads to nuclear exclusion (20). Some mutant p53 proteins that carry missense or linker insertion mutations within the protein coding region are also expressed in the cytoplasm of transformed cells (22, 23). To begin to understand the mechanism that controls p53 translocation we embarked on a study to characterize unique proteins that form a complex with p53 within the cytoplasm in vivo.

Utilizing a transformed rat embryo fibroblast cell line that expresses copious amounts of a recombinant temperature-sensitive mutant murine p53 (TSp53Val-135)1 in the cytoplasm at the non-permissive temperature (39 °C), a 92-kDa protein was observed to form a complex with TSp53Val-135 in vivo. p92 was purified to homogeneity and, by microsequence analysis, identified as hsp84, a member of the heat shock 90 family (hsp90). hsp90 is a molecular chaperone previously shown to bind a discrete set of intracellular proteins including inducible transcription factors such as steroid receptors and the dioxin receptor (24–26). To test if hsp84 binds p53 in a regulated fashion the cell line expressing TSp53Val-135 was incubated at the permissive temperature (32 °C), which leads to a well documented conformation shift within the p53 protein and promotes TSp53Val-135 nuclear translocation (23). Under these conditions little or no hsp84 was bound to p53. This study demonstrates that mutant p53 forms a complex with hsp84 in a spatial and/or conformation-specific manner.

EXPERIMENTAL PROCEDURES

Cell Culture and Harvesting—The A1 rat embryo fibroblast cell line was maintained and grown in 15-cm diameter dishes and split 1:5 upon confluency. After 2 days at 37 °C cells were incubated either at 32 °C or 39 °C for an additional 24 h and subsequently harvested and stored at −80 °C as described previously (27). For metabolic radiolabeling, cells were incubated with 4 ml of starving medium (methionine-free Dulbecco’s modified Eagle’s medium, 2% dialyzed fetal bovine serum) per dish at 39 °C. After 1 h the medium was replaced with 4 ml of fresh starving medium supplemented with 200 μCi of [35S]methionine as Tran35S-label (ICN). Cells were labeled for 5–6 h, rinsed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na2HPO4, 1.5 mM KH2PO4), centrifuged, and stored as cell pellets at −80 °C.

Preparation of the p53 Immunoaffinity Column—Monoclonal antibody PAB421 was purified as described previously (27) and dialyzed against PBS supplemented with 0.01% CaCl2 and 0.01% MgCl2. FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; HPLC, high performance liquid chromatography; MOPS, 4-morpholinopropanesulfonic acid.
against coupling buffer (100 mM acetic acid, 150 mM NaCl (adjusted to pH 5.5 with acetic acid or sodium hydroxide solution)) at 4 °C overnight with one change of coupling buffer (final dilution-fold was 1:40,000).

The antibody (4 mg/ml) was coupled to Ag-Hz-agarose beads (Bio-Rad) according to the manufacturer’s recommendations. The final concentration of antibody coupled to the column was 2.73 mg/ml resin with a coupling efficiency of 73%. Protein concentration was determined using the Bio-Rad assay dye with bovine serum albumin as a protein standard.

p92 Purification—A1 soluble lysate (4.5 mg/ml) from approximately 106 cells (280 × 15 cm plates) was applied to a 10-mI immunaffinity column equipped with a 2-mI precolumn as described previously (27) with some modifications. The loaded column was washed (1 column volumes each) with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40), SNNT (5% sucrose, 1% Nonidet P-40, 0.5 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA), RIPA (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), and again with lysis buffer. The p53-p92 complex was eluted after each pass with 4 column volumes of lysis buffer supplemented with 0.2 mg/ml epitope peptide. The peptide solution was circulated through the column for 1.5 h at a flow rate of 2.4 ml/min and collected. After acetone precipitation a total of 4 ml of 0.75 mg/ml protein in SDS sample buffer was loaded into each lane of a 10% polyacrylamide gel (15 cm × 29.5 cm × 1.5 mm) and run at 80 V (constant voltage). The gels were incubated with B/T Blv (protein stain) (Intermountain Scientific, Carlsbad, CA) for 1.5 min and destained for less than 30 min according to the manufacturer’s instructions. After rinsing with water the p92 bands were excised with a razor blade and stored at −20 °C. It was estimated that each band contained 0.5–0.7 μg by densitometry of the stained gels and comparison to known amounts of bovine serum albumin. Density of protein bands was measured on an Imaging Densitometer (Bio-Rad, model GS-670) equipped with Molecular Analyst software (version 2.0) run on a Macintosh Centris 650. To concentrate the p92 protein, a customized GS-670 equipped with Molecular Analyst software (version 2.0) run on a Macintosh Centris 650 was used to construct a slab gel with large wells (each well had dimensions 15 mm × 45 mm). Each well was loaded with 6–9 gel slices (each containing approximately 5 μg), each gel slice being cut into 5-mm segments by gelatin solution at 25°C (constant current). The p92 was stained and excised as described above. The final seven gel slices, each containing approximately 5 μg were combined into a single lane of another gel and run on the same apparatus under the same conditions. The p92 protein was electroblotted onto polyvinylidene fluoride Immobilon-P membrane (Millipore), trypsinized in situ, separated by reverse-phase HPLC, and sequenced as described previously (27, 28).

Immunoprecipitations and Western Blotting—Immunoprecipitation reactions were carried out as described previously (27) except when hsps84-specific antibody (PA3-012) was employed (Affinity BioReagents, Neshanic Station, N.J.). In such cases, a 50% protein A-Sepharose solution was prepared in MENGMN buffer (25 mM MOPS (pH 7.4), 2 mM EDTA, 150 mM NaCl, 1% glycerol, 20 mM sodium molybdate, 0.02% sodium azide). For each immunoprecipitation reaction, 10 μg of PA3-012 was combined with 50 μl of protein A-Sepharose solution, 100 μg of precloned lysate (in lysis buffer), and MENGMN buffer added to a final volume of 0.5 ml. Samples were rocked end over end overnight at 4 °C and the centrifuged pellets were washed twice with MENGMN buffer. The pellets were resuspended in 30 μl of SDS sample buffer, heated to 100 °C for 5–10 min, and loaded onto a denaturing 10% polyacrylamide gel. Protein transfer to Immobilon-P membrane was performed as described previously (29). For hsps84 detection the membrane was probed with 0.6 μg of PA3-012 in 10 ml of MENGMN buffer supplemented with 1% bovine serum albumin overnight.

Immunostaining (Immunofluorescence Staining)—A1 cells (106 cells in 2 ml of media) were grown on slide chambers (Lab-Tek Chamber model number 177380, Nunc Inc.) at 37 °C overnight and subsequently transferred to 39 or 32 °C for another 24 h. Cells were washed with ice-cold PBS**, fixed, and permeabilized with 100% chilled (−20 °C) acetone for 20 min. Slides were allowed to dry at room temperature and blocked with PBS** containing 10% gelatin and 0.05% Tween 20. Cells were incubated with purified PAb421 antibody and PA3-012, each at a final concentration of 10−15 μg/ml, for 1.5 h at 37 °C. Slides were washed five times with washing buffer (PBS** containing 0.05% Tween 20) with moderate shaking at room temperature. Cells were treated simultaneously with FITC-conjugated sheep F(ab)2 fragment anti-mouse IgG (Sigma) to detect p53 antibody and TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) to detect hsps84 antibody (each secondary antibody was used at a dilution of 1:100, final concentration was 1–2 μg per chamber) and shielded from light for 1.5 h at 37 °C. Cells were washed extensively with washing buffer and mounted with 10–15 μl of mounting buffer consisting of 13% Gelvatol (Ajax) and the anti-fading reagent 2.5% triethyl-enediamine (Sigma). Affinity purified non-immune rabbit IgG (Oncogene Science, New York) at a final concentration of 15 μg/ml was used as a negative control for hsps84 detection and the monoclonal antibody 2E1A (Oncogene Science) at a final concentration of 15 μg/ml was used as a negative control for p53 detection. An Olympus model A1BH microscope was used for conventional fluorescence microscopy viewed at 40 × power with a photo eye piece magnification of 2.5. Micrographs were taken on a mounted 35-mm camera with Fuji color 400 ASA film. An exposure time of 1 min was used when cells were viewed through the FITC filter and an exposure time of 20 s was used when cells were viewed through the TRITC filter. For confocal images a Carl Zeiss LSM 410 in vivo MOPS laser scan microscope connected to a PC computer was employed. Powerpoint software (version 4.0) was used for the layout and LSM software (version 3.7) was used to overlay the images. The images were printed on a Codonics NP-1600 photographic network printer.

Gel Filtration Chromatography—The soluble cytoplasmic fraction was prepared by the method of Kiyokawa et al. (30). Supernatant, representing the soluble cytoplasmic fraction was concentrated 4-fold on a Centricon-30 filter (Amicon) to a final volume of 250 μl, filtered through a 0.4-μm filter (Millipore) and injected onto a 24-ml Superose 6 column (Pharmacia Biotech) equilibrated in lysis buffer at 4 °C on a Gilson HPLC system. The flow rate of the column was 0.5 ml/min and 1-min fractions were collected. Fractions 11–37 were immuno-purified with PAB421 monoclonal antibody as described previously (27). Radioactivity in the protein bands was quantified by ImageQuant software (Molecular Dynamics) using volume integration. Background radioactivity in each lane was subtracted from p92 and p53 bands. The Gilson HPLC chromatography system included two pumps (model 306), a fraction collector (FC2089), a Rhodamine 700 injection valve, and was controlled with 712 HPLC System Controller Software run on a Compaq 486 PC.

RESULTS

Detection of a 92-kDa Protein Bound to p53 in Transformed Cells—To study endogenous p53-binding proteins in the cytoplasm we used a transformed rat embryo fibroblast cell line (A1) which expresses a high level of a murine temperature sensitive p53 protein, TSp53Val-135 in the cytoplasm at 39 °C,
the nonpermissive temperature (23, 31). Soluble lysate from A1 cells at 39 °C was applied to a Superose 6 gel filtration column (Pharmacia) and each 0.5-ml volume fraction (fractions 11–37) was collected and immunoprecipitated with PAb421 monoclonal antibody. A, immunoprecipitated p53-p92 complexes were separated on a 10% SDS-polyacrylamide gel and detected by PhosphorImager analysis (Molecular Dynamics). The positive control lane (+) represents an immunoprecipitation of the cytosolic fraction prior to concentration and application to the column. The fraction number is shown at the top of each lane. Fractions 11–13 (not shown) had no detectable protein. The relative ratio of radioactivity within p53 and hsp84 in each fraction (from 16 through 35) was quantified as described under “Experimental Procedures” and depicted at the bottom of each lane. A total of 9 × 10^7 cpm of trichloroacetic acid-precipitable radioactivity was applied to the column and 5 × 10^6 cpm was used for the immunoprecipitation in the positive control. B, levels of p53 (open squares) and bound p92 (filled squares) in each fraction were plotted as a function of fraction number. Molecular mass values used were as follows: thyroglobulin, 669 KDa; β-amylase, 200 KDa; bovine albumin, 66 KDa. The void volume (V_v) for the column was 8 ml (fractions 1–16) and was determined by the exclusion of the initial peak of blue dextran (Pharmacia Biotech). Gel filtration molecular size standards (Sigma) were individually run on the column at the same flow rate to establish standard K_v values.

**Fig. 2.** Partial purification of p53-p92 complex on a gel-filtration column. A1 cells were metabolically radiolabeled with [35S]methionine at 39 °C and the soluble cytoplasmic fraction was isolated as described under “Experimental Procedures.” The sample was applied to a Superose 6 gel filtration column (Pharmacia) and each 0.5-ml volume fraction (fractions 11–37) was collected and immunoprecipitated with PAb421 monoclonal antibody. A, immunoprecipitated p53-p92 complexes were separated on a 10% SDS-polyacrylamide gel and detected by PhosphorImager analysis (Molecular Dynamics). The positive control lane (+) represents an immunoprecipitation of the cytosolic fraction prior to concentration and application to the column. The fraction number is shown at the top of each lane. Fractions 11–13 (not shown) had no detectable protein. The relative ratio of radioactivity within p53 and hsp84 in each fraction (from 16 through 35) was quantified as described under “Experimental Procedures” and depicted at the bottom of each lane. A total of 9 × 10^7 cpm of trichloroacetic acid-precipitable radioactivity was applied to the column and 5 × 10^6 cpm was used for the immunoprecipitation in the positive control. B, levels of p53 (open squares) and bound p92 (filled squares) in each fraction were plotted as a function of fraction number. Molecular mass values used were as follows: thyroglobulin, 669 KDa; β-amylase, 200 KDa; bovine albumin, 66 KDa. The void volume (V_v) for the column was 8 ml (fractions 1–16) and was determined by the exclusion of the initial peak of blue dextran (Pharmacia Biotech). Gel filtration molecular size standards (Sigma) were individually run on the column at the same flow rate to establish standard K_v values.
p53 Copurifies with p92 on Gel Filtration Chromatography—If p53 and p92 form a complex in the cytoplasm then one might expect the two proteins to copurify from cells by other purification techniques. To test this, A1 cells were metabolically radiolabeled and the soluble cytoplasmic fraction was obtained through differential centrifugation. Microscopic inspection indicated that the nuclei were intact. The cytoplasmic fraction was applied to a gel filtration column and each fraction collected from the column was immunoprecipitated with the p53-specific antibody PAb421. The immunoprecipitated fractions were analyzed for p53 and p92 by SDS-polyacrylamide gel electrophoresis followed by PhosphorImager analysis. The result of this experiment is shown in Fig. 2A. p53 was detected in fractions 16 through 35 and p92 was detected in fractions 16 through 34. hsc70 protein appeared to coelute with these two proteins as well. Quantitative analysis revealed that the p53 to p92 ratio ranged from 5.4 to 9.4 in fractions 16 through 33. These values cannot be taken as molar ratios since the proteins were radiolabeled without consideration for turnover kinetics. Fig. 2B represents the elution profile of p53 and bound p92.

Comparision to molecular size standards suggests that the p53-p92 complex is distributed over a wide range of sizes. We detected a potential peak at 350 kDa (fractions 28–29) with larger complexes eluting near the void volume of the column (the exclusion limit for spherical proteins is 5,000 kDa on this column). It should be noted that the non-ionic detergent (Nonidet P-40) used in the buffer may alter the elution properties of the p53-p92 complexes (37). The experiment suggests that p53, hsc70, and p92 form complexes of heterogeneous size within the cell.

Identification of p92 by Sequence Analysis—To identify p92 approximately 30 μg of the protein was purified to homogeneity and sequence information was obtained from four internal p92 peptides. The sequences from the peptides was used to search for homologous sequences in the TBLAST E-mail server at the National Center for Biotechnology Information (38). The result of the search indicated that all four rat p92 peptides shared 100% identity with the putative protein sequence encoded by the murine heat shock protein 84 (hsp84) cDNA (Table I). hsp84 is a member of the heat shock 90 class of proteins (hsp90) previously observed to bind a wide variety of intracellular proteins (39). In mouse cells there are two isozymes of hsp90, named hsp84 and hsp86, that are 86% conserved at the amino acid level (40). To investigate if mouse hsp86 also shared a high degree of identity with p92, the p92 peptide sequences were optimally aligned with the hsp86 putative protein sequence. The identity shared between these p92 peptides and hsp86 ranged from 85 to 100%, suggesting that p53 can only form a complex with hsp84 and not hsp86. However, we cannot rule out the possibility that other peptides derived from p92 represent hsp86 sequences.

p53 Colocalizes with hsp84 in the Cytoplasm—If p53 forms a complex with hsp84 in the cell, one might expect these two proteins to be colocalized in the same area of the cell. To determine if this was the case, A1 cells growing at 39 °C were permeabilized and probed simultaneously with p53-specific
monoclonal antibody PAb421 and a polyclonal antibody generated against the well-conserved amino-terminal peptide of murine hsp84 (41). The cells were viewed by confocal microscopy using the appropriate filter to discern p53 staining from hsp84 staining (Fig. 3). As shown in panel A, p53 was detected in the cytoplasm, particularly close to the nuclear envelope in a punctate pattern. As depicted in panel B, the majority of hsp84 was also detected in the cytoplasm in a punctate pattern. The pattern of hsp84 staining is in agreement with previous studies on murine hsp84 in a hepatoma cell line (41). Panel C is an overlay of the images in panels A and B demonstrating the concordance of the staining patterns. This experiment suggests that the majority of p53 and hsp84 is colocalized in the same subcompartment within the cytoplasm. Upon close inspection of the micrographs it appears that a minor fraction of p53 and hsp84 colocalized in the nucleus as well.

p53 Conformation Change and/or Nuclear Localization Correlates with a Reduction of hsp84 Binding—At 39 °C TSp53Val-135 resides in the cytoplasm and adopts a conformation detected with PAb240 monoclonal antibody (22, 23, 42). At 32 °C p53 resides in the nucleus and adopts a conformation detected by a monoclonal antibody (PAb246) that recognizes a “wild-type like p53.” If hsp84 colocalized with p53 at 32 °C it would suggest that hsp84 can bind p53 independently of p53 conformation or location. Cells were plated at 37 °C and then either shifted to 39 °C to allow p53 to reside in the cytoplasm or to 32 °C to allow p53 to reside in the nucleus. The cells were subsequently fixed and processed for double-staining immunofluorescence studies as described in Fig. 3. The results of this experiment are shown in Fig. 4. Panel A indicates that the majority of p53 was located in the cytoplasm at 39 °C. As shown in panel B, at 32 °C the majority of p53 was located in the nucleus with some residual p53 remaining in the cytoplasm. Panel C shows that the subcellular location of hsp84 mimicked that of p53 at 39 °C. However, panel D demonstrates that hsp84 resided in the cytoplasm at 32 °C. Thus, p53 and hsp84 colocalized when p53 was incubated at 39 °C but not when the cells were incubated at 32 °C (p53 in nucleus). These results suggest that hsp84 binds p53 only at 39 °C, when the majority of p53 is in the cytoplasm.

Since hsp84 remained in the cytoplasm when p53 resided in the nucleus one might expect a lower concentration of the p53-hsp84 complex under these conditions. To test this hypothesis the relative level of p53 bound to hsp84 was determined when the cells were incubated at 39 or 32 °C. To prevent possible dissociation of the p53-hsp84 complex the immunopre-
At the permissive temperature p53 and hsp84 are not in a complex. A1 cells were incubated at 39 or 32°C for 24 h prior to preparation of soluble lysate from A1 cells. A, equal amounts of soluble lysate was immunoprecipitated with non-immune rabbit polyclonal antisera (w/v) or hsp84-specific rabbit polyclonal antisera (w/hsp84). The immunoprecipitated proteins were run on a 10% polyacrylamide gel and electroblotted onto polyvinylidene fluoride membrane. The membrane was cut horizontally at the 68-kDa prestained molecular mass standard. The upper half was probed with hsp84-specific rabbit polyclonal antisera and the lower half was probed with PAb421. Lane 1, lysate from A1 cells incubated at 39°C immunoprecipitated with w/v; lane 2, lysate from A1 cells incubated at 39°C immunoprecipitated with w/hsp84; lane 3, lysate from A1 cells incubated at 32°C immunoprecipitated with w/v; lane 4, lysate from A1 cells incubated at 32°C immunoprecipitated with w/hsp84. B, an equal amount of soluble lysate as in A was immunoprecipitated with negative control monoclonal antibody (PAb419) or p53-specific antibody (PAb421) followed by Western blot analysis using PAb421. Lane 1, lysate from A1 cells incubated at 39°C immunoprecipitated with PAb419; lane 2, lysate from A1 cells incubated at 39°C immunoprecipitated with PAb421; lane 3, lysate from A1 cells incubated at 32°C immunoprecipitated with PAb419; lane 4, lysate from A1 cells incubated at 32°C immunoprecipitated with PAb421. Migration position of prestained molecular mass standards (Life Technologies, Inc.) in kDa are represented on the left-hand side. Some staining due to the heavy chain of the control antibodies binding to the secondary antibody used in the Western blot analysis was detected in the negative control immunoprecipitations. Some p53 breakdown products below the major p53 protein are detected in the Western blot analysis in panel B. Immunoprecipitation reactions were carried out in the presence of molybdate to stabilize hsp90-target protein complexes (43). Immunoprecipitation of hsp84 followed by Western blot analysis is shown in Fig. 5A. Antibodies to hsp84 were able to immunoprecipitate an appreciable amount of p53 from cells incubated at 39°C but not at 32°C. These results are consistent with the subcellular colocalization studies presented in Fig. 4. Furthermore, the fact that hsp84-specific antibodies immunoprecipitated p53 confirmed that the two proteins form a complex. Importantly, the level of hsp84 appeared identical at both temperatures indicating that the lower level of p53-hsp84 complex detected at 32°C was not due to lower expression of hsp84. To exclude the possibility that the lower level of p53-hsp84 was due to a reduction in p53 at 32°C, the same extracts were immunoprecipitated using p53-specific antibody followed by Western blot analysis of p53. As shown in Fig. 5B, the p53 level appeared identical in both cell extracts. These data strongly suggest that the decreased level of p53 bound to hsp84 at 32°C was due to a reduction in the level of p53-hsp84 formed and not due to decreased cellular levels of p53 or hsp84. The results suggest that hsp84 may bind p53 in a spatial or conformation-dependent manner.

**DISCUSSION**

In this study we report the identification of a 92-kDa protein (p92) that forms a complex with a mutant murine p53 protein (TSp53Val-135) predominantly in the cytoplasm of transformed cells. p92 communoprecipitated with p53 using two monoclonal antibodies that recognize separate epitopes on p53. The protein was purified to homogeneity and the amino acid sequence information of four internal tryptic peptides derived from p92 demonstrated it was a homologue of murine hsp84, a member of the heat shock 90 class of proteins (40). The heat shock 90 gene family is well conserved; members of the same isozyme share over 80% identity between eukaryotes from Droso- phila to Homo sapiens. For the purpose of discussion the term hsp90 shall be used instead of hsp84 because in previous studies it was often not which isozyme of hsp90 bound its target proteins.

Gel filtration analysis showed that the p53-hsp90 complex is heterogeneous in size. The majority of the complex appeared to elute as a 350-kDa complex; but larger size complexes were also detected. Western blot analysis of the p53-hsp90 complexes separated by gel filtration chromatography confirmed the fact that the majority of the p53-hsp90 complex was 350 kDa in size. hsc70, another heat shock protein, appeared to copurify with the p53-hsp90 complex. This is not surprising since previous studies have shown that hsp70 (which is highly related to hsc70) is essential for the assembly of dimeric hsp90/progres- terone receptor complexes in vitro and remains bound to the receptor until it binds hormone (44). Other studies have shown that TSp53Val-135 from these same cells exists as a high molecular mass aggregate containing hsc70 and an uncharacterized 110-kDa protein, but no hsp90 (45). It is probable that the harsh purification procedures previously used stripped hsp90 from p53 since high ionic strength buffers can dissociate hsp90 from its target proteins (46).

p53 and hsp90 colocalized within the cytoplasm close to the nuclear envelope. At 39°C p53 is thought to form an altered conformation recognized by the PAb240 antibody (23). We find that the p53-hsp90 complex can be communoprecipitated with this antibody (see Fig. 1B). At the permissive temperature, 32°C, when TSp53Val-135 is in the nucleus, it does not express the PAb240 epitope, but instead, is recognized by another monoclonal antibody PAb246 (23). We detect little or no p53 bound to hsp90 at the permissive temperature and hsp90 appears to remain in the cytoplasm (see Figs. 4 and 5A). It is not clear if hsp90 binds p53 because of its conformation or because it resides in the cytoplasm. From our studies, the former explanation is favored because a low level of hsp90 and p53 are colocalized in the nucleus at the nonpermissive temperature (see Figs. 3 and 4). We are currently conducting experiments to clarify this issue.

This article complements a recent report showing that an antibiotic, geldanamycin, destabilizes mutant p53 expressed in human tumor cells (47). Geldanamycin disrupts macromolecular complexes containing hsp90 and leads to the destabilization of the hsp90 target protein (48–50). Our demonstration that hsp90 forms a physical complex with p53 suggests that the

---

2 I. B. Paz and J. Momand, unpublished data.
effect of geldanamycin on mutant p53 stability may be through its prevention of stable p53-hsp90 complex formation. Mutant p53 peptides generally have a half-life of 1–5 h while wild-type p53 has a half-life of 20 min or less (31, 51). The extended half-life of mutant p53 may be important in its transforming effect as a transforming protein by physically sequestering endogenous wild-type p53 (52–54). It is possible that hsp90 binding to p53 contributes to the increased stability of mutant p53 proteins.

Our observation that p53 can form a complex with hsp90 within the cytoplasm significantly extends a study in which p53-binding proteins were previously characterized in the breast cancer cell line T47D (55). This cell line expresses an endogenous mutant p53 protein in the cytoplasm as well as in the nucleus. Using a p53-specific antibody, a 90-kDa protein was observed to communoprecipitate with p53 exclusively from the cytoplasmic fraction. This protein had electrophoretic properties similar to hsp90, but the study lacked direct proof that the protein coprecipitating with p53 was hsp90.

We have identified hsp90 as a protein that forms a complex with mutant p53 predominantly within the cytoplasm of transformed cells. We have shown that hsp90 dissociates from p53 under conditions where p53 undergoes a conformational change and translocates to the nucleus. It is unclear if wild-type p53, under some circumstances, can also bind hsp90. If wild-type p53 is capable of adopting a PAB240+ conformation, it may form a complex with hsp90. It has been reported that peripheral blood cells from primary acute myeloid leukemia patients express wild-type p53 in a PAB240+ conformation (56). Furthermore, when wild-type p53 is translated in vitro in the presence of rabbit reticulocyte extract it can express a PAB240+ conformation (57). We are currently investigating the functional significance of the p53-hsp90 interaction and the possibility that hsp90 binds wild-type p53 expressed in the cytoplasm of human cancer cells.

Acknowledgments—We thank William S. Lane of the Harvard Microchemistry Facility for in situ proteolytic digestion of p92, separation of p92 peptides by reverse phase HPLC, and Edman chemical sequence analysis. We thank Dr. Bruce Kaplan at the City of Hope Peptide Synthesis Facility for synthesis and purification of the epitope peptide. We thank Dr. Robert B. Colvin at the Children’s Cancer Research Institute for his generous donation of plasmid of human cancer cells.

We thank Dr. Bruce Kaplan at the City of Hope Peptide Synthesis Facility for synthesis and purification of the epitope peptide. We thank Dr. Robert B. Colvin at the Children’s Cancer Research Institute for his generous donation of plasmid of human cancer cells.

REFERENCES
1. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. V., and Vogelstein, B. (1990) Science 249, 915–920
2. Finlay, C. A., Hinds, P. W., and Levine, A. J. (1989) Cell 27, 1083–1093
3. Livingstone, L., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tlsty, T. D. (1994) Nature 370, 795–799
4. Blondal, J. A., and Benchimol, S. (1994) Oncogene 9, 153–159
5. Ozbun, M. A., and Butel, J. S. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 6407–6411
6. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) Science 261, 703–707
7. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) Cell 75, 817–825
8. Parekh, B., and Reddy, B. D. (1994) Oncogene 9, 1601–1608
9. Alberts, B. S., Johnson, A. D., Lewis, J. W., Morgan, D. L., and Raff, M. C. (1989) Science 245, 809–818
10. Lee, S., Elenbaas, B., Levine, A., and Griffith, J. (1995) Cell 81, 1013–1020
11. Wu, J., Bayle, J., Elenbaas, B., Pavletich, N. P., and Levine, A. J. (1996) Mol. Cell. Biol. 15, 497–504
12. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. (1993) Nature 362, 849–851
13. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993) Nature 362, 847–849
