Vol. 184, No. 1, 1992
April 15, 1992

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 167-174

HSP70 BINDS SPECIFICALLY TO A PEPTIDE DERIVED FROM THE HIGHLY CONSERVED DOMAIN (I) REGION OF P53

Kelvin T. Lam[1] and Stuart K. Calderwood[2]

[1] The MGH Cancer Center, Harvard Medical School, Building 149, Charlestown, Massachusetts 02129

[2] Dana-Farber Cancer Institute and Joint Center for Radiation Therapy, Harvard Medical School, 50 Binney Street, Boston, Massachusetts 02115

Received February 28, 1992

Products of a number of mutant p53 genes bind with high affinity to members of the hsp70 family of chaperonin proteins, whereas wild type p53 lacks this type of association. Examination of the sequences of p53 genes from five different species enabled us to predict domains on p53 which may be involved in the association with hsp70 family members. A synthetic polypeptide (Pro-17-Gly) corresponding to the candidate hsp70 binding domain bound to in vitro translated hsp70 as determined by affinity chromatography and nondenaturing gel mobility shift assays. In addition, the Pro-17-Gly peptide competitively inhibited association between hsp70 and p53, an activity which was determined by immunoprecipitation with anti-p53 monoclonal antibody PAb240. The data indicate that p53 contains a hsp70 binding domain, which is located in a highly conserved region at the amino terminus of the protein, and may participate in the cellular function of wild-type p53 or in the transforming capacity of p53 mutants.

It is becoming generally accepted that the p53 tumor antigen, a nuclear phosphoprotein, functions as a tumor suppressor protein in the cell. The p53 gene is located on chromosome 17p13, and many colorectal carcinomas exhibit allelic deletions and/or point mutations in this region (1,2) which may be crucial for the expression of the malignant phenotype. A role for p53 as a tumor suppressor protein is supported by the finding that cells derived from certain human tumor biopsies express mutant p53 proteins. In addition, several lung cancer cell lines (3), leukemia T-cell lines (4) and some osteosarcoma cell lines (5,6) also contain point mutated p53 genes. Mutations that were detected in the p53 gene lie in regions that are conserved between species (7).

Many p53 mutations result in loss of tumor suppressor activity coupled with the acquisition by such p53 mutants of trans-dominant cell transforming

0006-291X/92 $1.50

capacity. Such transformation by p53 mutants is associated with the capacity of the mutant p53 to bind and sequester the product of the wild type allele in stable hetero-oligomeric complexes (7). The complexes containing wild type and mutant p53 proteins may also contain a member of the 70 kilodalton heat shock protein family (hsc70).

In the present report, we have investigated potential hsp70 binding sites in p53 by examination of the p53 gene for evolutionary conserved domains and for sequences susceptible to transforming mutation. A synthetic peptide conforming to the most likely domain (I) was made and examined for binding to hsp70. Our studies indicate that the p53-derived peptide binds to hsp70 and competes with the full-length p53 for hsp70 binding.

Materials and Methods

Cell line

RKT101-Y rat embryo fibroblasts were kindly provided by Dr. A. Levine (Princeton University). Cells were grown in Dulbecco's minimal essential medium supplemented with 10% bovine calf serum at 37oC in a 5% CO2/95% air atmosphere. For heat shock studies, cells were incubated for 12 min at 45oC in a water bath containing a circulator located inside a 5% CO2/95% air incubator.

Antibodies

Monoclonal antibodies that recognize the mutant p53 (PAb240) were generously provided by Dr. D. Lane (Imperial Cancer Research Fund, London). The polyclonal antibody that recognizes hsc70 was prepared against a synthetic peptide that comprises a highly conserved internal domain of hsp70 (Ab LTS-156) (8).

Plasmids and in vitro transcription/translation

The human hsp70 gene (9) was cloned into an in vitro transcription vector which contains the SP6 promoter and in vitro transcription carried out as described by the manufacturer (Promega).

Synthetic peptides and peptide-agarose affinity chromatography

The amino acid composition of the p53 peptide, Pro-Leu-Ser-Gln-Glu-Thr-Phe-Ser-Gly-Leu-Trp-Lys-Leu-Leu-Pro-Pro-Glu-Asp-Gly (Pro-17-Gly), was derived from mouse p53 cDNA sequence (figure 1) (10). A peptide conforming to this sequence was synthesized commercially (Multiple Peptide Systems, San Diego, CA) and purified by reverse-phase high-performance liquid chromatography. Before use in experiments, the peptide was desalted on 15cm Biogel P2 columns (Bio-Rad, Richmond, CA). For affinity chromatography studies, the peptide was coupled to Affi-Gel10 (Bio Rad), a cross-linked agarose gel to which is attached, by ten atom spacers, N-hydroxysuccinimide groups. Peptides were incubated with Affi-Gel10 at a concentration of 10mg / ml of resin for 4 hours at 4oC in 100mM MOPS, pH 7.5, 80mM CaCl2. Affinity resin was then quenched with 0.1M glycine and washed exhaustively to remove unbound peptide before use in affinity chromatography. For control experiments we used the protein kinase inhibitory peptide

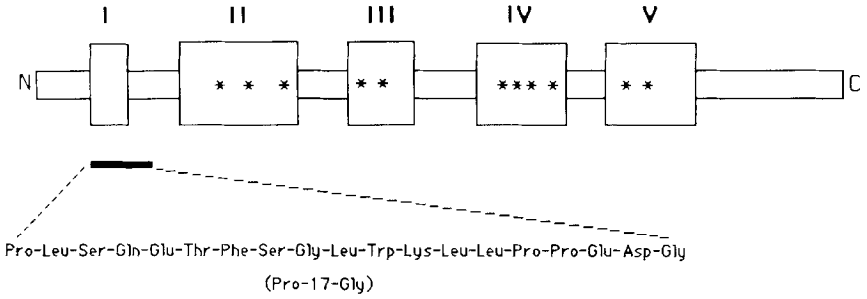

Figure 1
Sequence comparison of p53 tumor antigen from different species indicating the five highly-conserved domains (Lane & Benchimol, 1990). Point mutations (*) were detected predominantly in the conserved domains (Baker et al, 1989; Nigro et al, 1989; Takahashi et al, 1990; Chen & Haas, 1990; Diller et al,1990; Malkin et al, 1990), and a peptide (Pro-17-Gly) overlapping the first conserved domain, used in this study, is shown as a black bar.

Thr-18-Asp:(Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg -Asn- Ala-Ile-His-Asp) (Sigma, St. Louis, MO). This peptide was chosen because it is of similar size (20-mer) compared to the p53 peptide (19-mer). Peptides were dissolved in peptide binding buffer (PBB) (25mM Tris, pH 7.4; 150mM NaCl; 5mM KCl; 3mM MgCl2; 1mM DTT; 1mM PMSF).

Nondenaturing gel electrophoresis

The hsp70 associated protein complexes were separated by 4% nondenaturing gel electrophoresis for 2 hours at a constant voltage (100 V) in a standard Tris-glycine buffer system (8).

Immunoprecipitation

Immunopreciptation experiments were performed on lysates prepared from RKT101-Y cells. Cellular proteins were metabolically labeled with 35S-methionine-containing medium for 16 hours and cells then lysed by sonication in RIPA buffer (10mM Tris, pH 7.4; 150mM NaCl; 1.0% NP-40; 1.0% Deoxycholate; 0.1% SDS). The immunoprecipitation was performed essentially as described by Hinds, P.W. et al (16).

Results

In order to identify potential hsp70 binding sites on the p53 protein, we examined sequences from different species in order to locate evolutionarily conserved domains and mutation sites of known biological activity (7). Our rationale was that, if hsp70 binding is biologically important, the binding site should be located in a conserved region of the gene that is not susceptible to mutation.

To test whether the Pro-17-Gly peptide derived from p53 has the ability to bind to hsp70, we used Pro-17-Gly-agarose peptide affinity chromatography (figure 2). The peptide was covalently coupled to agarose beads, and in vitro translated, 35S-methionine radiolabeled hsp70 was allowed to bind to the peptide agarose beads. Figure 2 shows the profile of

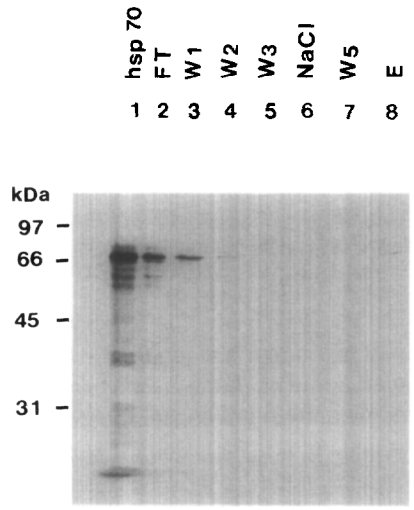

Figure 2
Electrophoretic analysis (12% SDS-PAGE) of protein fractions from p53 peptide (Pro-17-Gly) agarose affinity resin after incubation with in vitro translated hsp70. Pro-17-Gly was coupled to agarose beads as described in Materials and Methods and hsp70 (lane1) prepared by in vitro transcription/translation of the hsp70 gene was bound to the resin for 1hr at 4°C. Subsequent lanes indicate a flow through (FT) fraction (lane 2), three washes (W1,W2 and W3) with peptide binding buffer, a high salt (NaCl) wash with 1M NaCl containing PBB and a final wash (W5) with PBB. Hsp70 was eluted(E) from the washed resin with 500μM p53 peptide (lane 8).

proteins eluted from the peptide affinity column. Lane 1 represents the in vitro translated, 35S-methionine labeled hsp70 that was incubated with the peptide beads. Lane 2 shows the flow through product, which did not bind to the peptide beads. Lanes 3, 4 and 5 are three sequential washes with PBB, followed by high salt (1mM NaCl) wash (lane 6) and a final wash with PBB (lane 7), After the third PBB wash, no hsp70 was removed even by high-salt washing. hsp70 was, however, specifically eluted using the Pro-17-Gly peptide (lane 8). The association between hsp70 and p53 peptide was resistant to high salt (lane 6) suggesting high affinity binding between hsp70 and Pro-17-Gly. We were unable to detect hsp70 binding to a control resin which contained glycine cross-linked to Affi-Gel 10 agarose, indicating that hsp70 binds specifically to the immobilized Pro-17-Gly rather than non-specifically to the agarose support (data not shown).

We also examined the capacity of the peptide to competitively inhibit hsp70-p53 binding using immunoprecipitation as an assay. It has been shown previously that monoclonal antibody (PAb240) recognizes a domain between amino acids 156 and 214 on murine p53 (11), and therefore should not directly bind to Pro-17-Gly (amino acids between 13 and 30). We carried out co -immunoprecipitation experiments on lysates from RKT101-Y cells that

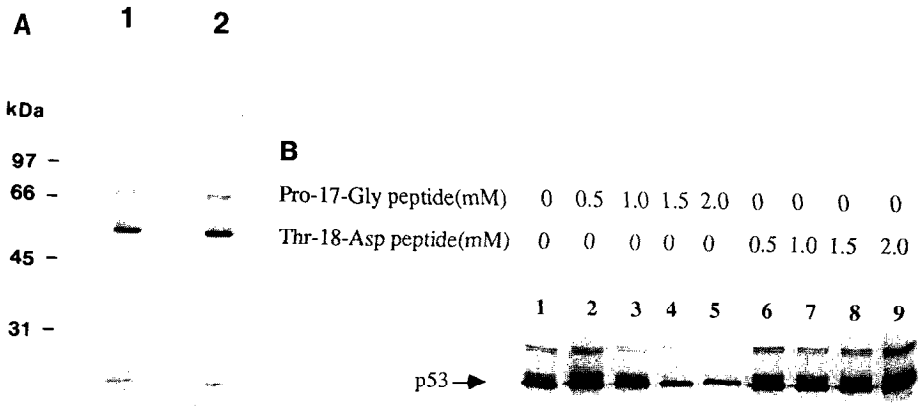

Figure 3

(A) Immunoprecipitation of complexes containing p53, hsp70 and hsc70 from RKT101-Y cell lysate using monoclonal antibody PAb240. Control cells (lane 1) and cells pre-heat shocked for 12 min at 45°C (lane 2) were incubated with 35S-methionine (25μCi/ml medium) for 12 hr at 37°C prior to lysis in RIPA buffer and immunoprecipitation as described in Materials and Methods. Immunoprecipitated proteins were analysed by 12% SDS-PAGE .

(B) Lysates prepared from cells previously heat shocked to induce hsp70 (as in legend to Fig3A) were incubated with increasing concentrations (from 0.5mM to 2.0mM) of either the p53-derived peptide Pro-17-Gly (lanes 2 to5) or a control peptide (Thr-18-Asp) (lanes 6 to 9) prior to immunoprecipitation analysis with PAb240 as described above. Immunoprecipitates were dissolved in elecrophoresis buffer and analysed by 12% SDS-PAGE.

express a mutant p53 gene. Experiments were carried out on cells that were either unstressed or which had been heat shocked 12hr previously to induce hsp70. The aim was to determine whether p53 interacts with both the constitutive (hsc70) and heat-inducible (hsp70) hsp70 isoforms. When non-heat shocked cells were used, hsc70 was shown to be co-immunoprecipitated with p53 by PAb240 (lane 1,figure 3A). Immunoprecipitation analysis of lysates from heat shocked cells indicated association between p53 and both hsp70 and hsc70 (lane 2).

To test whether Pro-17-Gly has the ability to competitively inhibit hsp70-p53 binding, the peptide was incubated with lysates from RKT101-Y cells expressing hsp70 and the p53-containing protein complexes were collected by immunoprecipitation with PAb240 (figure 3B). In the absence of added peptides, mutant p53 was associated with both heat shock proteins (lane1). Addition of Pro-17-Gly led to the dissociation of hsp70 from p53-containing complexes (lane5) in a dose dependent manner (lanes 2 to 5). However, incubation with a control synthetic peptide (Thr-18-Asp), of a similar size to Pro-17-Gly, failed to disturb the interaction between hsp70 and p53 (lane 6 to 9) suggesting that the effect of Pro-17-Gly on protein-protein association is sequence specific. We therefore concluded from

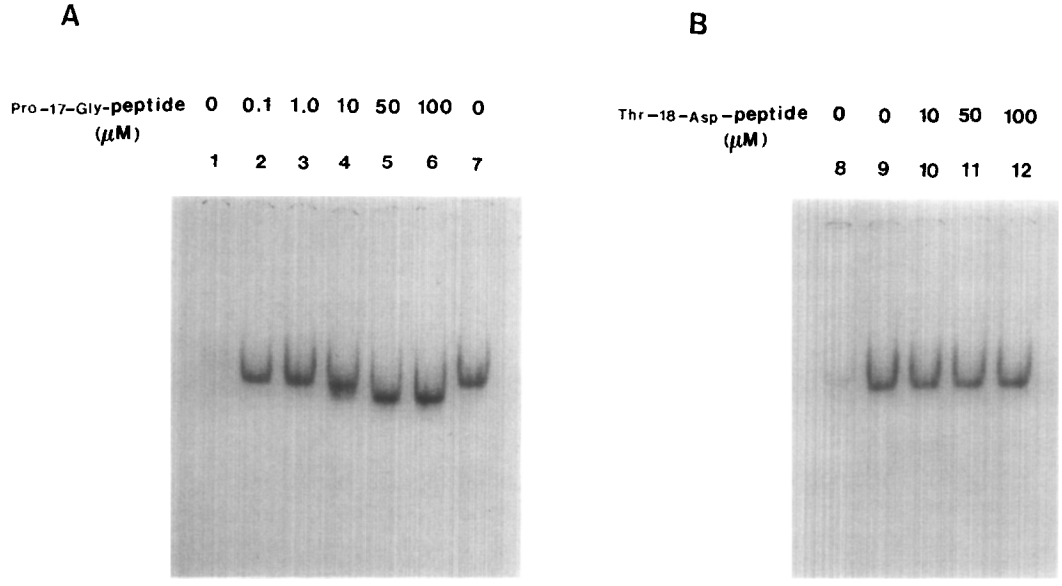

Figure 4

(A) Effect of peptides on the electrophoretic mobility of hsp70. Hsp70 labeled with ^{35}S-methionine was prepared by in vitro translation and analysed by non-denaturing gel electrophoresis. A control hsp70 sample (lane7) and hsp70 incubated with increasing concentrations (from 0.1μM to 100μM) of Pro-17-Gly (lanes 2-6) were subjected to 4% nondenaturing gel electrophoresis as described in Materials and Methods. Lane 1 contains a control sample of reticulocyte lysate to which no in vitro transcribed hsp70 mRNA was added and which thus contains no human hsp70. Cell lysates were incubated with Pro-17-Gly at 20°C for 1 hr prior to electrophoresis.
(B) The experiment was similar to the one described in the legend to figure 4A except that a control peptide (Thr-18-Asp) was added in increasing amounts (from 10μM to 100μM) to the in vitro translated products instead of Pro-17-Gly. The lysate mixtures were subjected to 4% non-denaturing gel electrophoresis.

figure 3 that the Pro-17-Gly peptide derived from p53 specifically inhibits the association between hsp70 and p53.

Hsp70-peptide interactions were also investigated using nondenaturing PAGE. We examined the capacity of Pro-17-Gly to alter the electrophoretic mobility of the in vitro translated hsp70 protein on 4% nondenaturing gels (figure 4A). Lanes 1 and 7 represent the translated products in the absence or presence of in vitro transcribed hsp70 mRNA. The peptide was added in increasing amounts (0.1uM to 100uM) to the in vitro translated hsp70 (lanes 2 to 6). At concentrations of 50uM Pro-17-Gly and above, the hsp70 protein complex migrated significantly faster than in controls (lane 7). To test the specificity of this effect, a second peptide (Thr-18-Asp) of a similar size to Pro-17-Gly peptide was added to the translated product and analyzed by the 4% nondenaturing gel electrophoresis (figure 4B). The addition of increasing

amounts of Thr-18-Asp to hsp70 (lanes 10-12) did not alter the migration pattern compared to controls containing no added peptide (lane 9). The induction of a shift in electrophoreic mobility of hsp70 by Pro-17-Gly probably thus indicates a sequence-specific interaction of hsp70 with the peptide, reinforcing our similar conclusions from the affinity chromatography and immunoprecipitation studies (Figs 2,3)

Discussion

In the present study we have demonstrated that a synthetic polypeptide (Pro-17-Gly) derived from the highly conserved domain (I) region of p53 has the ability to bind hsp70 using peptide affinity chromatography and nondenaturing gel mobility shift assays. In addition, Pro-17-Gly peptide competitively inhibited the association between hsp70 and p53. The data, therefore, indicates that Pro-17-Gly contains a binding site for hsp70. The 70 kDa heat shock proteins are members of a family of polypeptide chain binding proteins that recognize unfolded proteins and may function as chaperonins to facilitate formation of correctly folded protein (13). It is therefore possible that mutated p53 may have an unfolded or "incorrect" conformation that is recognized by heat shock proteins. Such a mutated p53 might be locked into the "incorrect" conformation due to the structural constraints imposed by point mutation. It is also possible, however, that hsp70 proteins associate with a consensus binding site on p53 rather than recognizing mutant p53 gene products as aberrantly folded. It has been shown that hsc70 specifically recognizes a consensus sequence in a highly conserved region of the clathrin light chain, a major substrate for the protein (14). This hsc70 binding sequence in the clathrin light chain is rich in proline and glycine residues presumbly in same respects the Pro-17-Gly sequence in p53 (figure 1) which also contains proline and glycine and could also constitute be a specific hsp70 recognition site analogous to the hsc70 binding domain on the clathrin light chain.

The biological implications of the interaction of p53 with hsp70 are unknown. Hsp70 may have a regulatory role in p53 metabolism and could, for example, participate in the assembly and disassembly of oligomeric complexes containing p53, which may be an intermediates in p53 activity in the cell. Formation of high molecular weight complexes containing p53 may be crucial in the cellular function of the protein. The highly conserved carboxy terminus of hsp70 (12) is thought to be involved in binding to other substrates, and it is conceivable that p53 may bind to the same region. However, the precise binding region of hsp70 to p53 is yet to be determined and we are presently carrying out a deletion mutational analysis of the murine hsp70 gene to map the p53 binding domain of hsp70, using Pro-17-Gly as a substrate.

In conclusion therefore we have shown in the present study that a synthetic peptide derived from the first highly conserved region of p53 binds to the seventy kilodalton heat shock protein. This peptide also inhibits the association of mutant p53 with hsp70 proteins, suggesting that the peptide contains the hsp70 binding domain on p53.

Acknowledgments

This investigation was supported by NIH Grants CA44940 and CA47407, and K.T.L. was supported by postdoctoral fellowship training grant number CA09078.

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