Activation of the latent DNA binding function of human p53 protein by the bacterial Hsp70, DnaK, represents a unique reaction in which a heat shock protein can interact with a native protein to affect its function. We have localized a likely DnaK interaction site on native human p53 tetramers to a motif flanking the COOH-terminal casein kinase II and protein kinase C phosphorylation sites. Murine p53 is less efficiently activated by DnaK, which has permitted a search for factors that might cooperate in p53 activation by DnaK. We show that optimal activation by DnaK may be dependent upon the phosphorylation state of murine p53, in particular, modification of p53 at the cdc2 phosphorylation site by point mutation decreases the extent of activation by DnaK. Additionally, the monoclonal antibody PAb241, binding in the vicinity of the cdc2 phosphorylation site, is able to activate the specific DNA binding function of p53. This has led us to propose a second regulatory motif flanking the tetramerization domain of p53 that cooperates with factors binding at the negative regulatory domain in the extreme COOH terminus.

Heat shock proteins comprise a highly conserved set of polypeptides taking part in a number of protein folding processes such as the prevention of aggregation or premature protein folding, acceleration of the rate of polypeptide folding into the native state, and assembly or disassembly of oligomeric protein complexes (1, 2). The levels of heat shock proteins are often increased after cellular stress, as observed in many disease states including ischemia, inflammation, oxidant and tissue injury, cardiac hypertrophy, fever, and cancer (3). Members of the Hsp701 and Hsp60 protein family have been the most extensively studied heat shock proteins. Hsp70 isoforms are highly conserved between prokaryotic and eukaryotic cells, with multiple and distinct members present in yeast and humans. Hsp70 proteins all have a highly conserved ATP-binding domain and a more divergent peptide-binding domain which may dictate substrate specificity (4–6). Hsp70 protein function is also linked to an obligate partner heat shock protein, often the Hsp40 or DnaJ homologue, which can aid in directing Hsp70 protein to its substrate and in catalyzing turnover (7, 8).

Although much research on the biochemical function of heat shock proteins has been centered on the putative role these proteins play in their interaction with non-native conformations of heat-denatured target proteins, there is a growing body of evidence implicating Hsp70 isoforms in interacting with native protein substrates. One of the first physiological roles observed for a heat shock protein family member was in the Hsp70-dependent dissociation of clathrin from coated vesicles (9). The reaction mechanism is now known to involve first the binding of the DnaJ-box containing protein Auxilin to the native clathrin lattice and subsequent recruitment of Hsc70 (10). Auxilin is subsequently released while Hsc70 promotes dissociation of clathrin-clathrin interactions causing triskelion release. A second Hsp70 homologue clearly involved in a physiological interaction with native protein complexes is the Escherichia coli DnaK protein. DnaK is essential for normal cellular physiology of E. coli (reviewed in Ref. 11) and it is also required for initiation of DNA replication from the E. coli chromosomal origin of replication (12) and of bacteriophage λ (13) and P1 origins (14). Its role during bacteriophage λ DNA replication also requires the Hsp40 homologue, DnaJ protein, and involves their interaction with and dissociation of a native, oligomeric protein-protein complex at the λ origin of replication, thus catalyzing initiation of DNA synthesis. Similarly, DnaK and DnaJ proteins can together aid in dissociation of inactive RepA dimers into active monomers promoting initiation of P1 phage DNA replication. Importantly, DnaK and DnaJ proteins have been recently shown to bind not only to denatured polypeptide substrates, but to native bacteriophage replication proteins RepA and λ-P protein (15, 16). These results suggest that heat shock protein family members can recognize specific determinants on native polypeptides, although the specific domains involved in these interactions have not yet been defined.

Intriguingly, members of the Hsp70 protein family have been shown to interact with the human tumor suppressor proteins p53 and pRb (17), although the significance of these interactions is not clear. Earlier studies have shown that mutant p53, but not wild-type p53, bound to Hsp/c70 in tumor cell lines (18–21). Recent data also demonstrated that human Hsp40, the eukaryotic homologue of DnaJ, is also part of the complex of mutant p53 and Hsp/c70 (22). Mutant p53 protein in tumor cell lines is often in an unfolded or denatured conformation as defined by expression of a normally hidden antibody epitope, PAb240 (23, 24), suggesting that the heat shock protein holoen-
zyme complexes may be recognizing unfolded domains on mutant p53 or may be promoting the unfolding of mutant p53 protein.

Recently, a novel biochemical reaction was observed for an Hsp70 homologue; DnaK alone can mimic the action of mammalian protein kinases by interacting with and activating the latent sequence-specific DNA binding activity of wild-type human p53 protein (25) without changing its tetramerization state, suggesting that the activation reaction has an allosteric component (26). DnaK protein cannot re-activate heat-denatured p53 (27), indicating that the mechanism of activation of latent p53 is not related directly to the interaction of DnaK protein with a denatured substrate, as observed previously with RNA polymerase (28). The mechanism whereby DnaK protein can mimic the effects of protein kinases to activate latent p53 has not been addressed. We have localized a likely interaction site on human p53 protein for DnaK and compared the binding of eukaryotic members of the Hsp70 family to a same domain. Notably, three distinct isoforms of the stress 70 protein family cannot substitute for DnaK protein in the human p53 activation reaction. Further analysis of DnaK activation of murine p53 has led us to define two COOH-terminal domains on p53 which appear to be involved in the mediation of DnaK protein activation. These results underscore the likelihood that DnaK protein activation of latent p53 is similar to that of protein kinases and define a two-component biochemical system for studying the function of DnaK protein as it interacts with and changes the activity of a native protein substrate.

MATERIALS AND METHODS

Reagents—Human Hsp70 and Hsc70 were expressed in E. coli and purified as published elsewhere (7). Recombinant hamster Gpr78 expressed in E. coli and was obtained from StressGen Corp. Non-biotinylated peptides were obtained from Pfizer.

Purification of DnaK—A DnaK over-expressing strain was obtained from Dr. Maciej Zylicz, Gdansk, Poland. The cells were grown shaking in LB media at 30 °C to an OD_{600,nm} of 0.5, when they were quickly heat-shocked by 2-fold dilution in prewarmed (55 °C) LB media. Subsequently, the incubation was continued for another 3 h at 42 °C. Cells were pelleted by centrifugation and resuspended in 100 mM HEPES, pH 7.6, to an O.D. equivalent to 1.5. The cell suspension was lysed for 45 min on ice by adding KCl to 0.25 M, DTT to 2 mM, lysozyme to 0.5 mg/ml, benzamidine to 1 mM, and leupeptin to 1 mg/ml. After centrifugation, the supernatant was filtered through a 0.45-m filter, and then loaded onto a Q50-Sepharose column at a protein (mg):resin (ml) ratio of 10:1. Bound protein was eluted using a linear gradient from 0.05 to 1 M KCl (in buffer B, containing 10% glycerol, 20 mM HEPES, pH 7.5, 5 mM DTT, and 0.1 mM EDTA). Fractions containing DnaK were dialyzed against a buffer containing 10% sucrose, 20 mM imidazole, pH 7.0, 20 mM MgCl₂, 20 mM KCl, 5 mM DTT, 1 mM benzamidine, and leupeptin to 1 mg/ml (buffer A). Dialyzed protein was applied to an ATP-agarose column (Sigma A-2767) in buffer A at a protein (mg):resin (ml) ratio of 20:1. After washing in buffer A containing 1 M KCl and equilibration in buffer B, DnaK was eluted in buffer A containing 10 mM ATP.

Purification of Latent Human p53 from E. coli—Recombinant human p53 was expressed in BL21 E. coli cells at room temperature using a T7 expression system (29), in which latent p53 tetramers were purified from soluble lysates (25). p53 was purified by a modification of a published protocol. Briefly, human p53 was purified using HiTrap Heparin-Sepharose (Hi-Trap, Pharmacia Biotech Inc.), phosphocellulose (P-11, Whatmann), and Superose-12 (Pharmacia) column chromatography.

Purification of Murine p53 Protein—Murine wild-type p53 was expressed in E. coli BL21 cells as described for human p53 (30). Murine wild-type p53 and the point mutant Ala-309 were also expressed in Spodoptera frugiperda cells (59 cells) as described elsewhere (27). The protein was purified using heparin-Sepharose (Hi-Trap, Pharmacia Biotech Inc.) with a linear KCl gradient from 0.1 to 1 M KCl in buffer B.

Peptide Binding Assay—Peptides used in this assay were purified and obtained from Chiron Mimotopes and contain a biotin group at the N terminal linked to the amino acid sequence SSAG. Peptides are 15-mer representing the linear amino acid sequence of human or murine p53. DnaK (1.5 μM) was incubated with 0.05, 0.5, and 5 μM peptide in reaction buffer (15% glycerol, 25 mM HEPES, pH 7.5, 50 mM KCl, 5 mM DTT, 0.02% Triton X-100, 10 mM MgCl₂, 1 mM ATP) for 30 min at 30 °C. Reactions were run on a native 8% polyacrylamide gel at 50 V for 3 h, then blotted onto nitrocellulose as described (30) and the peptides were detected with streptavidin-conjugated peroxidase (Sigma) and ECL (Amersham).

Phosphorylation of Latent p53—p53 protein (12 ng) was incubated in 10 μl of a buffer (10% glycerol, 10 mM MgCl₂, 20 mM HEPES, pH 7.6, 0.1 mM ATP, 0.1 mM EDTA, 5 mM DTT, 0.1% Triton X-100) with casein kinase II from rabbit muscle (31) or recombinant human casein kinase II obtained from Boehringer Mannheim (0.1 milliunit). Incubations were performed for the indicated times at 30 °C and reactions were added to 10 μl of DNA binding buffer containing radioactive target DNA as described below.

DNA Binding Assay—Binding conditions are described in detail elsewhere (25). Briefly, the DNA binding buffer contained 20% (v/v) glycerol, 50 mM KCl, 40 mM HEPES, pH 7.5, 0.05 mM EDTA, 5 mM DTT, 0.1% Triton X-100, 10 mM MgCl₂, and 1.0 mg/ml bovine serum albumin. A double-stranded oligonucleotide representing the specific p53 consensus site (25, 32) was end-labeled with [γ³²P]ATP and used with a 20-fold excess of supercoiled nonspecific, non-labeled competitor DNA (pBluescript SK⁻, Stratagene). Incubations of p53 with DnaK or peptide were performed for 30 min at 30 °C in a 10-μl reaction buffer. Then 10 μl of DNA binding buffer containing 5 ng of labeled p53 consensus site and 100 ng of pBluescript were added to the reaction mixture, incubated on ice for 30 min, loaded on a 4% native polyacrylamide gel, and run at temperatures from 4 °C to 12 °C. For the quantification of the radioactive signals a PhosphorImager (Molecular Dynamics) was used.

RESULTS

DnaK Targets the COOH-terminal Regulatory Site of Human p53—DnaK can activate latent, wild-type, and some mutant forms of p53 protein for sequence-specific DNA binding (31). Although the mechanism is undefined, it is clear that the tetramerization status of p53 is preserved after activation, indicating that DnaK does not dissociate tetramers into smaller units (26). DnaK activation of p53 also requires energy in the form of heat, as the reaction cannot proceed at 0 °C (see below). In addition, DnaK partner proteins DnaJ and GrpE are not required for this interaction, reducing the likelihood that substrate targeting and turnover, putatively associated with GrpE and DnaJ function, are required for DnaK function in this system. Given the simplicity of the two-component activation reaction driven by DnaK protein, studying the mechanism of p53 interaction with DnaK may not only provide insight into how p53 protein activity is altered by interacting proteins, but it may give insight into the mechanism whereby DnaK interacts with a native protein substrate.

Modification of a COOH-terminal negative regulatory domain on latent p53 has been shown to trigger the activation of DNA binding by other proteins. Binding of the monoclonal antibody PAB421 or phosphorylation by either casein kinase II, protein kinase C, or cd2/cyclins can activate latent p53 (25, 33–36), whereas the monoclonal antibody ICA-9 binding to the casein kinase II site can reverse the activation (37) (Fig. 7). As the target site for activation of p53 by DnaK has not been determined, we investigated a possible interaction of DnaK with the COOH-terminal regulatory domain.

A series of synthetic peptides was screened for the ability to interfere with the activation of human p53 DNA binding by DnaK. Reactions were divided into two stages by first incubating p53 and DnaK in the absence or presence of synthetic peptides at 30 °C and then assaying for sequence-specific DNA binding at 0 °C. Synthetic peptide 379–393 derived from the COOH terminus of p53 competed effectively with DnaK (Fig. 1A, lanes 8–10 versus lane 3). The concentration of DnaK in the

5 S. Hansen, C. A. Midgley, D. P. Lane, B. C. Freeman, R. I. Morimoto, and T. R. Hupp, unpublished observations.
Interaction of Hsp70 Isoforms with p53

**Fig. 1. Peptides derived from the COOH-terminal regulatory domain of human p53 compete with DnaK activation.** A, competitive binding experiments with peptide 379–393 and all three activating proteins: lane 1, p53, no activator; lane 2, p53 + PAb421; lane 3, p53 + DnaK; lane 4, p53 + casein kinase II; lanes 5–7, p53 + PAb421 with 1 μg, 0.1 μg, and 0.01 μg of peptide; lanes 8–10, p53 + DnaK with 1 μg, 0.1 μg, and 0.01 μg of peptide; and lanes 11–13, p53 + casein kinase II with 1 μg, 0.1 μg, and 0.01 μg of peptide. B, competitive binding experiments using peptide 369–383 which contains the 421 epitope: lanes 1–3, p53 + PAb421 with 1 μg, 0.1 μg, and 0.01 μg of peptide; lanes 4–6, p53 + DnaK 1 μg, 0.1 μg, and 0.01 μg of peptide; and lanes 7–9, p53 + casein kinase II 1 μg, 0.1 μg, and 0.01 μg of peptide. C, peptide competition in DnaK-dependent activation reactions. The indicated peptides were incubated in reactions assembled with DnaK and p53 at concentrations of 1, 0.1, 0.01, and 0.001 μg (lanes 2–5, peptide 379–393; lanes 6–9, peptides 374–388; and lanes 10–13, peptide 369–383). Lane 1 contains DnaK and p53 incubated at 30 °C for 30 min. The lack of activation of latent p53 on ice by DnaK (lane 14) demonstrates the temperature-dependence of the activation reaction. After peptide addition and incubation in the reaction, DNA binding was assayed as indicated under “Materials and Methods.” The position of the p53-DNA complexes are indicated.

The inability of a flanking peptide harboring the PAb421 antibody epitope (40) (peptide 369–383) to compete with DnaK activation indicates that peptides at stoichiometric concentrations do not in general inhibit the biochemical function of DnaK in this reaction (Fig. 1B, lanes 4–6). Peptide 369–383 is, however, biochemically active as it did inhibit p53 activation by PAb421 (Fig. 1B, lanes 1–3). Peptide 374–388 was also effective in competing with DnaK activation of p53 (Fig. 1C, lanes 6–9 versus lane 1). Together, these data indicate that amino acids 389–393 and 374–378 do not contribute to DnaK specificity while amino acids 379–388 play an important role in p53 recognition by DnaK (Fig. 7). Thus, this suggests that one important step in DnaK activation of p53 involves an interaction with a motif situated in between the casein kinase II and protein kinase C phosphorylation sites.

**DnaK binds directly to synthetic peptides derived from the p53 COOH terminus**—We examined whether the synthetic peptides derived from the COOH terminus of p53 were inhibiting activation of p53 protein by DnaK due to direct binding to DnaK. A slightly different subset of peptides was chosen for this analysis in order to define a minimal site of interaction. DnaK and the respective peptides were co-incubated at 30 °C, bound peptide was separated in a native polyacrylamide gel assay, and analyzed by Western blot via streptavidin binding to the biotinylated peptides (Fig. 2). DnaK did not interact with peptide 366–380, but showed weak binding to peptide 371–385, thus amino acids 381–385 define an important component of the DnaK binding site. Peptide 376–390 formed a stoichiometric complex with DnaK and exhibited the strongest binding to DnaK. The peptide 379–393 bound weaker to DnaK, as the presence of the last three acidic amino acids in the peptide may reduce the stability of complex formation with DnaK. Therefore, the last few amino acids do not contribute to the DnaK binding site, but rather seem to reduce binding. This is consistent with the fact that DnaK is a more potent activator of latent p53 protein when the final four amino acids are removed (41) and that peptide 379–393 has a slightly reduced inhibitory activity toward DnaK in comparison with peptide 374–388 (Fig. 1C, lane 4 versus lane 8). Together, these results indicate that DnaK can form a stable and stoichiometric complex with specific p53 peptides and refine assignment of the DnaK interaction site on p53 protein to amino acids 381–388 (Fig. 7).

**Interaction of Human Hsc70 Protein with p53 COOH-terminal Peptides**—Bacterial DnaK belongs to the heat shock protein 70 family and is very homologous to its human members (Table 1). Although Hsp70 homologues can exhibit quite distinct substrate specificities (6), peptides that bind to bacterial
DnaK with a high affinity contain some similar hydrophobic properties that match the consensus binding sequence for some mammalian heat shock proteins (4, 42), suggesting the possibility that some conservation of substrate specificity may exist. As such, we investigated whether mammalian Hsp70 isoforms can also bind directly to peptides derived from the COOH-terminal regulatory domain of human p53. Hsp70 family members studied include Grp78, Hsc70, and Hsp70 (see below).

The constitutively expressed Grp78/BiP is a resident protein of the endoplasmic reticulum and a member of the glucose-regulated protein family (43). An affinity panning approach of peptide libraries in bacteriophages has identified an optimal heptameric motif for the molecular chaperone BiP with a high content of aromatic and hydrophobic amino acids (4, 44). Recombinant hamster Grp78 (BiP), however, did not show any binding to our COOH-terminal p53 peptides (Table I). The 70-kDa heat shock cognate protein, Hsc70, is a constitutive member of the Hsp70 family found in the nucleus and cytosol, just like stress-induced Hsp70 (1). Human Hsp70 and Hsc70 proteins have been well characterized biochemically with respect to their ability to interact with denatured protein substrates (7). Although human Hsp70 protein did not bind to the COOH-terminal p53 peptides (Table I), human Hsc70 protein exhibited strong binding to the same peptides as observed with DnaK (Fig. 3). However, Hsc70 protein binding to the COOH-terminal p53 peptides was less avid than that observed for DnaK, which has been inferred previously, based on the relative ability of a COOH-terminal p53 peptide to compete with bovine Hsc70 and DnaK protein binding to unfolded lactalbumin (6).

The functional significance of this conserved interaction of DnaK and Hsc70 with the COOH-terminal p53 peptide is not yet clear. Evidence that mammalian Hsp/c70 proteins may modulate the p53 pathway comes from the observation that overexpression of Hsc70 protein can suppress focus formation of rat fibroblasts induced by mutant p53 plus ras and myc (45) and mammalian Hsc70/Hsp70 binds to mutant p53 synthesized in reticulocyte lysates unless the COOH-terminal 28 amino acids have been removed (46). Our data indicate that one interaction site for human Hsc70 protein may reside in the p53 protein COOH terminus and that human Hsp70 does not interact similarly. Despite the similarity of Hsc70 and DnaK in binding to COOH-terminal human p53 peptides, Hsc70 was not able to substitute for DnaK in activating latent human or murine p53 for DNA binding (data not shown). This suggests that: 1) another factor may be required to function in concert with Hsc70 to activate p53 protein; 2) the reduced affinity of Hsc70 is sufficient to preclude activation of p53 protein; 3) that DnaK protein has at least one other recognition site on p53, and 4) DnaK activation is not directly related to binding to the COOH-terminal region.

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### Table I

| Heat shock protein | Similarity to DnaK | Identity to DnaK | Binding to p53 COOH-terminal peptides |
|-------------------|--------------------|-----------------|--------------------------------------|
| DnaK              | 100                | 100             | +                                    |
| Human Hsc70       | 66.8               | 49.0            | ++                                  |
| Human Hsp70       | 66.9               | 50.1            | +++                                 |
| Human Grp78/BiP   | 69.2               | 51.2            | +/−                                  |
| Human Grp75       | 75.0               | 60.4            | –                                    |

* ND, not determined.

### Figure 3

**Binding of human Hsc70 to human p53 synthetic peptides.** 1.5 μM human Hsc70 was incubated with 0.05, 0.5, and 5 μM of the respective COOH-terminal biotinylated peptides of human p53. Protein bound to the peptides was detected with streptavidin-peroxidase and ECL following a native gel electrophoresis and blot onto nitrocellulose. DnaK (1.5 μM) incubated with equivalent amounts of peptide 376–390 were run on the same gel as an internal standard for the strength of the interaction.

### DnaK Binds with a Lower Affinity to COOH-terminal Peptides Derived from Murine p53–Murine p53 is 85.8% homologous to human p53 and both proteins include well described functional features like the DNA binding domain in the central region and a COOH-terminal tetramerization domain (47). Both proteins can activate transcription of target genes containing p53 binding sites (48) and they can both function as transcription factors in yeast (49). Human and murine p53 also form hetero-tetramers when co-translated (50). The cellular human proteins TBP (TATA-binding protein) and bhm2 (human double minute 2) also bind murine p53 (51, 52). However, although murine and human p53 have conserved their COOH-terminal cdc2/cyclin, protein kinase C, and casein kinase II phosphorylation sites, there is a striking degree of divergence in the region of human p53 implicated in binding to DnaK protein (Fig. 7). Peptides from the murine p53 COOH terminus bind much weaker to DnaK protein than the peptide from the human p53 sequence (Fig. 4), suggesting that this region does harbor a weaker DnaK interaction site. There are four amino acid changes in murine p53 compared to the human DnaK binding site (Fig. 7) and based on the Hsp70 isoform peptide binding specificity defined by Fourie et al. (6), the Leu to Thr, Pro to Val, and Thr to Lys amino acid substitutions in the COOH terminus of murine p53 may be sufficient to reduce DnaK binding. However, a similarity could be detected in terms of the relative affinity of binding pattern of murine COOH-terminal peptides; peptide 368–382 which localizes between the protein kinase C and casein kinase II phosphorylation sites showed a more pronounced binding than the neighboring peptides. If the COOH terminus is a major interaction site of p53 protein for
DnaK, then the reduced ability of DnaK to bind to murine p53 peptides should also be reflected in a reduced specific activity of DnaK in its activation of latent murine p53 protein. Addition of increasing concentrations of DnaK resulted in only a very weak activation of the DNA binding activity of latent murine p53 (Fig. 5), under conditions where PAb421 gave potent activation. These results are consistent with the hypothesis that the COOH terminus of latent human p53 protein harbors a DnaK recognition motif.

Mechanism of DnaK Activation of Murine p53—To confirm whether the COOH terminus of p53 harbors a DnaK recognition motif, the ability of DnaK to activate latent murine p53 derived from insect cells was also tested. Although murine latent p53 from E. coli was not activated by DnaK, latent murine p53 from insect cells was very efficiently activated in vitro by DnaK (Fig. 6A). As insect cells contain the three conserved kinases known to activate p53 (including cdc2/cyclin, protein kinase C, and casein kinase II) and as rat p53 produced in insect cells is heavily phosphorylated at the cdc2 and casein kinase II phosphorylation sites (33), it is likely that in vivo phosphorylation of murine p53 protein synthesized in insect cells may account for the increased activation seen with DnaK. Such a “priming” of latent human p53 protein by a kinase has been shown previously to render the latent, phosphorylated tetramer more receptive to activation by weak activators (26).

If this “priming” of murine p53 protein by in vivo phosphorylation at the cdc2 or casein kinase II sites was responsible for the ability of DnaK to activate latent murine p53, then dephosphorylation of the protein would presumably reduce the extent of activation by DnaK protein. One way to address this is to dephosphorylate in vitro the purified p53 protein. However, protein phosphatase dephosphorylation at the cdc2 and casein kinase II sites is very inefficient in vitro,3 precluding such an analysis. Instead, we chose to produce a cdc2 serine to alanine point mutation in the murine p53 protein which would preclude phosphorylation at this site in vivo. The murine p53Ala-309 mutant protein was significantly (50–65%) less active than wild-type p53 after incubation with DnaK protein (Fig. 6). This indicates a concerted synergistic action between binding of a factor at the COOH-terminal regulatory domain and phosphorylation at the cdc2 site. In fact, PAb421 was still efficient in activating insect cell-derived murine p53 protein mutated at this site (Ala-309).

Identification of a Second Regulatory Domain on p53 whose Modification Contributes to Activation—These data suggest that a second regulatory domain exists in the COOH terminus of p53, whose modification can lead to p53 activation. Since the binding of monoclonal antibodies often mimics the action of cellular factors and can regulate protein function in vitro (37, 61), we investigated the activity of latent murine p53 after incubation with the monoclonal antibody PAb241, whose epitope resides within amino acids 288–297 (54) and is close to

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3 R. Fahraeus and T. R. Hupp, manuscript in preparation.
the regulatory role played by this second domain, the monoclonal antibody PAb241 also activates p53 protein for DNA binding. Activated by DnaK if it is assembled and phosphorylated in recombinant insect cell expression systems. One site of phosphorylation that may act at the initiation codon, which predicts a 387-amino-acid protein. Within the DnaK binding site on human p53, there are four amino acid changes in the same site on p53. (The sequence of murine p53 was obtained from the Swiss protein data base and it is assumed that the second ATG is used as the initiation codon, which predicts a 387-amino-acid protein.) Within the DnaK binding site on human p53, there are four amino acid changes in human p53 which reduce the binding affinity of DnaK and lower the activation extent of murine p53 by DnaK. However, murine p53 is efficiently activated by DnaK if it is assembled and phosphorylated in recombinant insect cell expression systems. One site of phosphorylation that may act in concert (positive cooperation) with activators binding to regulatory domain I, is the cdc2 phosphorylation site (amino acid 309). Consistent with the regulatory role played by this second domain, the monoclonal antibody PAb241 also activates p53 protein for DNA binding.

DISCUSSION

Definition of an Hsc70 Binding Site on p53—The role of heat shock proteins in the p53 regulatory pathway it is not fully understood and various reports have suggested that an interaction between the Hsp70 protein family and p53 may be specific and of significance. Initial studies suggested a link with mutated p53, as in tumor cell lines, Hsp70 proteins immunoprecipitates with a fraction of mutant, but not wild-type p53 protein (18). In addition, overexpression of Hsc70 can suppress focus formation of cell lines induced by mutant p53 protein (45). The binding of rabbit Hsp70 from reticulocyte lysates to p53 protein requires the last 28 amino acids of murine mutant, not wild-type p53 (46), but these data did not establish whether this COOH-terminal region can directly bind to Hsp70 isoforms or if p53 conformation is altered by the deletion thus precluding Hsp70 binding.

More recent studies have shown that the p53 protein, induced by heat treatment of cells with a wild-type p53 gene, localizes in the cytoplasm, where it forms a complex with Hsc70 (55). The data presented in our study localizes one putative binding site of human Hsc70 to the extreme COOH-terminus of native wild-type p53 and is based on direct binding of peptides derived from the COOH-terminus of p53. We have, however, not established a stable and direct interaction of Hsc70 protein with native p53 protein (data not shown). The inability of Hsc70 protein to activate latent p53 may also imply that an obligate co-factor or protein(s) may be required to function in concert with Hsp70. Hsp40, a mammalian homologue of DnaJ, has been shown to form a complex with Hspc70 and mutant p53 (22). However, inclusion of human Hsp40 into the DNA binding reaction, together with Hsc70 did not lead to the activation of latent p53 (data not shown).

A physiological role for the interaction of p53 protein with heat shock proteins and whether a transient interaction with an Hsp70 family member in vivo is sufficient to activate p53 protein remains to be established. This latter hypothesis finds support from the observation that mutating all known regulatory phosphorylation sites on human p53 does not alter its biological function in human cells (57), suggesting that some other factor may be involved in p53 activation in cells. A second interesting question to address is whether the Hsc70 interaction with mutant p53 in tumor cells is a result of the altered conformation of the mutant protein or whether the mutant conformation is a consequence of the interaction with and unfolding by Hsc70 and its associated partner proteins. With one putative binding site of human Hsc70 localized on human p53 and the observation that human Hsp70 may not be involved in this process, this site can now be specifically targeted in vivo to approach these questions.

Definition of a DnaK Binding Site on p53—The bacterial heat shock protein DnaK is a potent activator of latent human p53 and allosteric class of mutant p53 proteins in vitro (25, 31). Since other activators (like PAb421, protein kinase C, and casein kinase II) also target the extreme COOH-terminal regulatory domain of p53 and another activator (cdc2/cyclin kinases (36)) targets a site flanking the tetramerization domain, we tried to determine if the site of interaction with DnaK was also in this region. We have identified synthetic peptides derived from the extreme COOH-terminal negative regulatory domain of p53 which compete effectively with DnaK activation of p53, suggesting that the COOH-terminal regulatory site of p53 contains an important interaction site for DnaK. We were unable to show effective inhibition of DnaK activation of latent p53 by synthetic peptides derived from the amino-terminal region of p53 at micromolar levels observed using carboxy-terminal peptides (data not shown), suggesting that the COOH-terminal regulatory site may contain the major interaction site for DnaK. A combination of the peptide inhibition studies (Fig. 1) and the direct peptide binding studies (Fig. 2) has led to the refinement of the DnaK binding site as amino acids 381–388 on p53. However, we have not yet shown a stable complex between DnaK and native, wild-type p53 protein, but if the interaction is transient or relatively weak, stable complexes may not exist.

The specificity of DnaK-peptide binding has been studied before, using a peptide display library (5). Short peptides with internal hydrophobic and NH₂-terminal positively charged residues were shown to be beneficial for binding by DnaK, whereas negatively charged amino acids only bound poorly. The peptide KKLMPFT, which corresponds to the DnaK binding site on p53, has been shown to compete efficiently with the binding of reduced and carboxymethylated lactalbumin (RCMLA) to DnaK (6). Indeed, by changing the second amino acid to a tyrosine (KYLMFPFT) the binding of bovine Hsc70, which only bound moderately to the original peptide, and the binding of bovine BIP, which did not bind at all to the original peptide, changes dramatically to reach the same degree as binding to DnaK. In murine p53 the sequence is changed in that particular region to KKMVFYK, which reduces the affinity of DnaK to the target sequence. Thus, the exchange of three hydrophobic residues and the introduction of a positive COOH-terminal charge reduces the affinity, but do not abolish critical amino acids for DnaK binding. However, unphosphorylated murine p53 produced in bacteria is not efficiently activated by DnaK,
unlike human p53 (Fig. 5), suggesting that mutation of these amino acids may effect the affinity of DnaK interaction with the native p53 tetramer.

**Mechanism of Activation of Mutant p53 by DnaK**—Whereas human and murine p53 are very similar on a structural level, they may respond differently to post-translational modifications that target the COOH-terminus. The carboxyl-terminal serine 392 site of human p53, which is phosphorylated by casein kinase II in vivo and in vitro, does not contribute to wild-type human p53’s biochemical and biological growth suppressor activity (41, 58). On the other hand, a mutation of the casein kinase II site of murine p53 inactivates its ability to suppress growth in rodent cell lines (59). Multiple evolutionary changes in amino acids between human and murine p53 in the extreme COOH-terminus suggest that this region of p53 has evolved slightly distinct controlling mechanisms despite having conserved the cdc2/cyclin, protein kinase C, and casein kinase II phosphorylation sites.

The activation of murine p53 from insect cells by DnaK represents a new model system, which helps to describe the mechanism of activation for DNA binding of murine p53. The activation of murine p53 by DnaK appears to be similar to the model proposed previously for activation of human p53 by phosphorylation (26), in which phosphorylation of latent human p53 in close proximity to the cdc2 site of phosphorylation (26), in which phosphorylation of latent human p53 in close proximity to the cdc2 site of phosphorylation (26), can be activated by weak activators, like peptides. Similarly, DnaK is a weak activator of unphosphorylated, murine p53, which may respond differently to post-translational modifications of the cdc2 site of phosphorylation.