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A New Member of the hsp90 Family of Molecular Chaperones Interacts with the Retinoblastoma Protein during Mitosis and after Heat Shock

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A gene encoding a new heat shock protein that may function as a molecular chaperone for the retinoblastoma protein (Rb) was characterized. The cDNA fragment was isolated by using the yeast two-hybrid system and Rb as bait. The open reading frame of the longest cDNA codes for a protein with substantial sequence homology to members of the hsp90 family. Antibodies prepared against fusions between glutathione *S*-transferase and portions of this new heat shock protein specifically recognized a 75-kDa cellular protein, hereafter designated hsp75, which is expressed ubiquitously and located in the cytoplasm. A unique LxCxE motif in hsp75, but not in other hsp90 family members, appears to be important for binding to the simian virus 40 T-antigen-binding domain of hypophosphorylated Rb, since a single mutation changing the cysteine to methionine abolishes the binding. In mammalian cells, Rb formed complexes with hsp75 under two special physiological conditions: (i) during M phase, when the envelope that separates the nuclear and cytoplasmic compartments broke down, and (ii) after heat shock, when hsp75 moved from its normal cytoplasmic location into the nucleus. In vitro, hsp75 had a biochemical activity to refold denatured Rb into its native conformation. Taken together, these results suggest that Rb may be a physiological substrate for the hsp75 chaperone molecule. The discovery of a heat shock protein that chaperones Rb identifies a mechanism, in addition to phosphorylation, by which Rb is regulated in response to progression of the cell cycle and to external stimuli.

Many studies have shown that the retinoblastoma protein (Rb) plays vital roles in cell cycle progression and cellular differentiation (reviewed in references 8 and 45). For example, Rb-deficient mouse fibroblasts grow faster and with less dependence on extracellular growth signals than cells identical except for expression of functional Rb. In addition, Rb-deficient tumor cells lose their growth advantage and are no longer tumorigenic in nude mice after the cells are reconstituted with the wild-type *RB* gene (27). A role for Rb in negatively regulating progression of the cell division cycle from G_1 to S phase has been clearly established. Overexpression of Rb in cells by microinjection or transfection inhibits cell cycle progression at mid-G_1 (20, 25). In vivo, overexpression of human Rb in transgenic mice results in dose-dependent growth suppression of the organism during embryonic and postnatal development (3). Mice that express no functional Rb fail to survive embryonic stages; they die during midgestation with defects in neurogenesis and hematopoiesis characterized by the failure of certain key neurons and erythrocyte precursors to exit the cell cycle and differentiate terminally (12, 30, 33, 34).

Regulation of Rb is mostly posttranslational and occurs by phosphorylation, dephosphorylation, and association with transcription factors and other proteins (45). Rb is phosphorylated and dephosphorylated during progression of the cell cycle (5, 9, 14). Hypophosphorylated forms predominate in G_0 and G_1, and progressive phosphorylations on serine and threonine residues occur in mid-G_1, in S, and near the G_2-to-M transition (9, 19). The phosphorylation events are closely tied to external signals. Upon receiving signals for proliferation, kinases are activated to phosphorylate Rb and release critical factors, such as E2F-1, that promote progression from G_1 to S (41, 48). The best candidates for proteins that directly phosphorylate Rb are cyclin-dependent kinases (37). In particular, cyclin D-cdk4 and cyclin E-cdk2 are the most likely candidates because they are active in G_1 (15, 18, 32). Dephosphorylation, probably by protein phosphatase 1 (2, 16), begins during anaphase and continues stepwise until completion later in mitosis (38, 39). Thus, as cells move into G_1, dephosphorylated Rb regains its growth-inhibitory functions as it presumably resumes its native conformation. Clearly, kinases and phosphatases play critical roles in regulating Rb function.

Rb functions through its interaction with other cellular proteins (36). All Rb-associated proteins reported to date bind the hypophosphorylated form of Rb. In addition to interacting with E2F-1 during G_1, hypophosphorylated Rb can bind to a number of DNA tumor virus oncoproteins, including simian virus 40 (SV40) large T antigen and adenovirus E1a (13, 54), when they are present within virally infected cells. Whereas hypophosphorylated Rb in G_0 or G_1 functions effectively to sequester E2F-1 and prevent progression of the cell cycle to S phase when growth signals are absent, its association with oncoproteins has the opposite effect. The oncoproteins bind tightly to the same domains used by many other Rb-associated cellular proteins; they thereby functionally inactivate Rb and allow unrestricted or deregulated progression of the cell cycle to committed phases.

Rb apparently also has a positive role in many cell differentiation processes (8). Rb-deficient cells and cells infected with tumor viruses, besides having deregulated cell cycle progression, have deficits in differentiating fully. Failure to exit from the cell cycle and to differentiate terminally is a characteristic

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of malignant cells as well as cells in Rb-deficient mouse embryos. For mouse embryos deficient in Rb, several reports have indicated that a large portion of the peripheral erythrocytes retain their nuclei and never mature (30, 33). At the same developmental stage, certain neuronal cells in the hindbrain and ganglia fail to develop into proper, mature neurons; they migrate to their usual positions but continue to divide when they should differentiate (33, 34). Possible mechanisms by which Rb might positively regulate differentiation have begun to be explored. Rb can bind to MyoD, a transcription factor important for muscle differentiation, and Rb-deficient myocytes fail to exit from the cell cycle in culture (21, 47). In addition, Rb binds to NF-IL6, a transcription factor important for differentiation of monocytes into macrophages, and directly activates NF-IL6 transcriptional activity (7). Rb also appears to be essential for adipocyte differentiation: fibroblasts deficient in Rb fail to respond to the hormones that induce differentiation into adipocytes. Reintroduction of an *RB* gene and reexpression of Rb in these cells restores their ability to differentiate like wild-type cells. Rb apparently functions by activating members of the C/EBP family of transcription factors, which are essential for adipocyte differentiation (5a). Recent discoveries of interactions between Rb and cofactors of the glucocorticoid hormone receptor (50) have further broadened the importance of Rb in general cell metabolism and in response to extracellular signals for proliferation, arrest, or differentiation.

In this communication, we describe the characterization of a heat shock protein that interacts with Rb specifically. The protein is one of the two dozen novel clones identified previously to interact with Rb in vitro and in *Saccharomyces cerevisiae* (16, 49). This heat shock protein has the ability to facilitate refolding of Rb in vitro and may act as a molecular chaperone for Rb in the cell under certain conditions. These results indicate that a heat shock protein, in addition to kinases and phosphatases, may provide another layer of regulation under specific conditions in which Rb needs to be properly folded or renatured.

MATERIALS AND METHODS

RNA blotting analysis. mRNA was prepared from cells as described previously (49). Five micrograms of each RNA was denatured in 50% formamide–2.2 mM sodium borate buffer (pH 8.3), separated by 1% agarose gel electrophoresis, and then transferred to a Hybond membrane (Amersham). Northern (RNA) hybridization was performed with appropriate probes as described previously (49).

Cloning and sequence analysis of C41 cDNAs. Cloning of cDNAs for Rb-associated proteins has been described previously (16). Two additional libraries were used for further screening: a λYES human lymphocyte cDNA library and a human fibroblast H1262 cDNA library (10). All inserts from isolated cDNA clones were subcloned into pBSK (Stratagene) for further analysis. DNA sequencing was performed by the dideoxynucleotide termination method (46). Sequences were analyzed with the computer program DNASTAR (DNASTAR, Madison, Wis.).

Plasmid construction and expression of fusion proteins in *Escherichia coli*. The glutathione *S*-transferase (GST) fusion systems were used to generate fusion proteins (51). Three different expression plasmids were constructed: GST-C41 (encoding amino acid residues 428 to 649), GST-C41N (encoding residues −24 to 252), and GST-C41FL (containing the full-length C41 cDNA). Expression of fusion proteins was performed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG), to a final concentration of 0.1 mM, to an exponentially growing bacterial culture at 30°C. After a 1-h incubation, bacteria were collected and lysed as described previously (49). Fusion proteins were purified with glutathione-agarose beads.

Antisera were raised in female BALB/c mice injected subcutaneously with 100 μg of GST-C41 and GST-C41N bound to glutathione beads in 75 μl of sterile phosphate-buffered saline (PBS) (0.9% saline). Mice were boosted with 100 μg of either GST-C41 or GST-C41N beads after 2 weeks and again after 2 months. For immunoprecipitation, the antisera were preabsorbed with an excess of the GST protein bound to glutathione-coated agarose beads for 60 min at 4°C before use.

Site-directed mutagenesis. To mutate cysteine 472 in C41-hsp75 to methionine, a 42-mer oligonucleotide (from codon 467 to 480) containing the mutation changing TGC to ATG at codon 472 was synthesized and used as a primer for PCR to generate the DNA fragment containing the mutation as described previously (24). The mutated DNA fragment was then substituted for the corresponding fragment of the wild-type C41 cDNA to construct pBSK-C41C472-M. The mutated C41 cDNA was then expressed either in a GST vector or in a yeast pSE1107 vector as described below.

[^{35}S]methionine metabolic labeling and in vitro transcription-translation. About 5×10^6 CV1 cells were labeled with [^{35}S]methionine (100 μCi/ml) for 2 h, and cell lysates were prepared for immunoprecipitation. The longest C41 cDNA in pBSK was used as template for in vitro transcription-translation, employing a TNT kit and the instructions provided by the supplier (Promega).

Cell lysate preparation, in vitro binding assay, and Western blotting (immunoblotting). Yeast cell extracts were prepared by growing 5 ml of cells to stationary phase under the appropriate selection conditions. Cells were then pelleted by centrifugation and lysed by being boiled in loading buffer with glass beads for 30 min. Aliquots representing equivalent cell numbers were then separated on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). CV1 cell extracts were prepared by lysing approximately 10^7 cells in Lysis 250 buffer (50 mM Tris [pH 7.4], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of antipain per ml), subjecting extracts to three freeze-thaw (liquid nitrogen-37°C) cycles, and clearing by centrifugation ($15{,}000 \times g$, 2 min, room temperature). Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon membranes. For analysis of mouse tissues, various organs from 2-month-old mice were pulverized under liquid nitrogen with a mortar and pestle. Proteins were then prepared as described previously (3).

In vitro binding assays were performed as previously described (10). Extracts made from 2×10^6 WR2E3 cells (6) were incubated with beads containing 2 to 3 μg of GST or GST fusion proteins in Lysis 150 buffer (which is identical to Lysis 250 buffer except that it contains 150 mM NaCl). Complexes were washed extensively with Lysis 150 buffer, boiled in loading buffer, and separated by SDS–7.5% PAGE. Gels were transferred to Immobilon membranes and probed with an anti-Rb monoclonal antibody, 11D7. Following addition of an alkaline phosphatase-conjugated secondary antibody, bound Rb was visualized with 5-bromo-4-chloro-3-indolylphosphate toluidinium and nitroblue tetrazolium (provided by Promega, Madison, Wis.).

The yeast two-hybrid system. A series of *RB* deletion mutants was cloned in frame with sequences for the Gal4 DNA-binding domain present on the expression vector pAS1, as previously described (16). In a second expression plasmid, pSE1107, C41 was joined to the Gal4 transactivation domain, creating pSE-C41. *S. cerevisiae* Y153 was cotransformed with these plasmids. Cotransformants were assayed for their ability to activate transcription of the *lacZ* gene, and the resulting β-galactosidase activity was measured. β-Galactosidase activity was determined by the colony lift method and quantitated with the chlorophenol red-β-D-galactopyranoside (CPRG) assay, as previously described (16).

Cell fractionation. The procedures to separate membrane, nuclear, and cytoplasmic fractions were adapted from those previously published (1, 35). All three fractions were then assayed for Rb and hsp75 by immunoprecipitation as described above. Aliquots of each fraction were also incubated with glutathione-agarose beads, which was followed by separation with SDS-PAGE and staining with Coomassie blue. GST was thus identified as a cytoplasmic marker.

Synchronization of cycling cells. The synchronization scheme for T24 cells has been described previously (9). Briefly, cells were arrested at G_0 or G_1 by growing them to saturation such that further division was contact inhibited. These arrested cells were then trypsinized and seeded at 3×10^6 cells per 10-cm-diameter plate in fresh medium. Three plates were collected for each time point as G11 (11 h after seeding), G23, etc. To obtain cells in M phase, cells released from contact inhibition for about 24 h were incubated with medium containing nocodazole (0.4 μg/ml). Mitotic cells were collected by gently shaking them off the dishes 12 h later. The synchronized cells were then used for protein analysis.

Coimmunoprecipitation. Coimmunoprecipitation was performed by a method previously described (16), with minor modifications. Synchronized cells (5×10^6) were lysed in cold Lysis 250 buffer. After a brief spin, the supernatant was diluted with an equal volume of lysis buffer (without salt). To each clarified supernatant was added 5 μl of monoclonal antibody 11D7 (anti-Rb) or anti-C41 mouse polyclonal antibody. Antigens and antibodies were incubated together for 1 h before the addition of Dynabeads M-450 (Dynal; 25 μl). After another 1-h incubation, the beads were collected and washed three to five times with Lysis 150 buffer. The beads were then boiled in SDS loading buffer for immunoblotting analysis.

Immunostaining. Cells grown on coverslips in tissue culture dishes were washed in PBS and fixed for 30 min in cold absolute methanol or 4% formaldehyde in PBS. Both fixatives resulted in the same pattern of immunostaining. In some cases, 0.5% Triton X-100 was added directly to the fixative in order to remove some soluble proteins, thereby accentuating fluorescent structures inside the cell (40). After being treated with 0.05% saponin in water for 30 min and extensively washed with PBS, the cells were blocked in PBS containing 10% normal goat serum. A 1-h incubation with a suitable antibody diluted in 10% goat serum was followed by three washes and then by another 1-h incubation with a fluorochrome-conjugated secondary antibody. Colocalization of $p110^{RB}$ and hsp75 was performed with a polyclonal rabbit anti-$p110^{RB}$ antibody, 0.47, mixed with anti-C41 mouse polyclonal antibody. The respective antigens were visual-

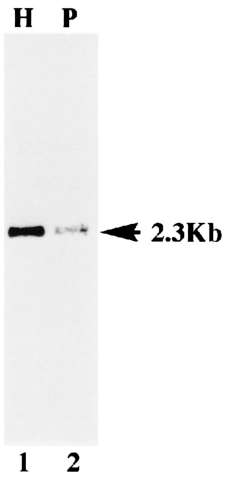

FIG. 1. Identification of C41 mRNA. About 5 μg of oligo(dT)-selected RNA from HeLa cells (H) (lane 1) or from human placenta (P) (lane 2) was analyzed. The RNA blot was hybridized with a ^{32}P-labeled, 0.8-kb fragment from the C41 cDNA and then autoradiographed. A single 2.3-kb band was detected in each lane.

ized with goat anti-rabbit immunoglobulin G (IgG) conjugated to Texas Red and goat anti-mouse IgG conjugated to fluorescein isothiocyanate. After being washed extensively in 0.5% Nonidet P-40, cells were further stained with the DNA-specific dye 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Permafluor (Lipshaw-Immunon, Inc.). Ektachrome P1600 was used when pictures were taken from a standard fluorescence microscope (Axiophot photomicroscope; Zeiss).

Denaturation, refolding, and partial trypsin digestion. The procedures for denaturation, refolding, and partial trypsin digestion experiments were as described previously (53) with modifications. Briefly, GST-RBS, a GST-p56^{RB} fusion protein (400 μg/ml), was denatured with 6 M guanidine hydrochloride in buffer A (20 mM phosphate buffer [pH 7.6], 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol) for 4 h at room temperature. The denatured protein was then diluted 10-fold with buffer A, and the guanidine-HCl was removed by Centricon 30 (Amicon) filtration according to the supplier's instructions. Refolding of denatured GST-RBS (0.2 μg/μl) was initiated by incubation with either GST-C41FL or GST in buffer B (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.6], 2 mM ATP, 5 mM $MgCl_2$, and 1 mM dithiothreitol) at room temperature for 30 minutes. TPCK (*N*-tosyl-L-phenylalanyl chloromethyl ketone)-treated trypsin was then added to the reaction mixture at 4 ng/μl, and the reactions were continued at 37°C for 45 min. The partial proteolytic fragments were separated by SDS–12.5% PAGE and immunoblotted with anti-B peptide antibody (23). The immunoreactive bands were detected by using an alkaline phosphatase detection system as described above.

RESULTS

Isolation of clone C41, encoding a protein with sequence homology to hsp90. C41 is one of the clones isolated from a screening for proteins able to bind N-terminally truncated p56^{RB} by a method involving the yeast two-hybrid system, as described previously (16). A 0.8-kb insert of C41 was used as a probe to perform RNA hybridization. As shown in Fig. 1, a single 2.3-kb mRNA was detected in HeLa cells or placental tissue. The 0.8-kb insert thus must be a partial clone. By screening several cDNA libraries, we found the longest clone to be 2.2 kb. The complete sequence of this clone is shown in Fig. 2. The longest open reading frame, from the most 5′ end to near the 3′ end, encodes a putative protein consisting of 698 amino acids. Since there is no methionine found in the N-terminal region, an alternative to the typical AUG initiation codon (4) must be used in this gene. Twenty-six additional clones isolated from several different libraries all ended with similar regions or were shorter than 2.2 kb. Significant homology was found between the protein putatively encoded by C41 and the hsp90 family of proteins (Fig. 3) (44). The similarity is located primarily in two noncontiguous regions. Assuming the 50th codon of the full-length cDNA to be the initiation site (see below), the first region, from amino acid residue 38 to 234 of the C41 gene product, has 43.4% similarity to the region containing amino acids 22 to 218 of hsp90a. The second region of the C41 product, from amino acid 235 to 646, has 26.6%

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GAGCTGCGGGCGCTGCTGCTGTGGGGCCGCCGCCTGCGGCCTTTGCTGCGGGCGCCGGCGCTGGCGGCCGTGCCGGGAGGAAAACCAATTCTGTGTCCTCGGAGGACCACAGCCCAGTTG   120
 E  L  R  A  L  L  L  W  G  R  R  L  R  P  L  L  R  A  P  A  L  A  A  V  P  G  G  K  P  I  L  C  P  R  R  T  T  A  Q  L
GGCCCCAGGCGAAACCCAGCCTGGAGCTTGCAGGCAGGACGACTGTTCAGCACGCAGACCGCCGAGGACAAGGAGGAACCCCTGCACTCGATTATCAGCAGCACAGAGAGCGTGCAGGGT   240
 G  P  R  R  N  P  A  W  S  L  Q  A  G  R  L  F  S  T  Q  T  A  E  D  K  E  E  P  L  H  S  I  I  S  S  T  E  S  V  Q  G
TCCACTTCCAAACATGAGTTCCAGGCCGAGACAAAGAAGCTTTTGGACATTGTTGCCCGGTCCCTGTACTCAGAAAAAGAGGTGTTTATACGGGAGCTGATCTCCAATGCCAGCGATGCC   360
 S  T  S  K  H  E  F  Q  A  E  T  K  K  L  L  D  I  V  A  R  S  L  Y  S  E  K  E  V  F  I  R  E  L  I  S  N  A  S  D  A
TTGGAAAAACTGCGTCACAAACTGGTGTCTGACGGCCAAGCACTGCCAGAAATGGAGATTCACTTGCAGACCAATGCCGAGAAAGGCACCATCACCATCCAGGATACTGGTATCGGGATG   480
 L  E  K  L  R  H  K  L  V  S  D  G  Q  A  L  P  E  M  E  I  H  L  Q  T  N  A  E  K  G  T  I  T  I  Q  D  T  G  I  G  M
ACACAGGAAGAGCTGGTGTCCAACCTGGGGACGATTGCCAGATCGGGGTCAAAGGCCTTCCTGGATGCTCTGCAGAACCAGGCTGAGGCCAGCAGCAAGATCATCGGCCAGTTTGGAGTG   600
 T  Q  E  E  L  V  S  N  L  G  T  I  A  R  S  G  S  K  A  F  L  D  A  L  Q  N  Q  A  E  A  S  S  K  I  I  G  Q  F  G  V
GGTTTCTACTCAGCTTTCATGGTGGCTGACAGAGTGGAGGTCTATTCCCGCTCGGCAGCCCCGGGGAGCCTGGGTTACCAGTGGCTTTCAGATGGTTCTGGAGTGTTTGAAATCGCCGAA   720
 G  F  Y  S  A  F  M  V  A  D  R  V  E  V  Y  S  R  S  A  A  P  G  S  L  G  Y  Q  W  L  S  D  G  S  G  V  F  E  I  A  E
GCTTCGGGAGTTAGAACCGGGACAAAAATCATCATCCACCTGAAATCCGACTGCAAGGAGTTTTCCAGCGAGGCCCGGGTGCGAGATGTGGTAACGAAGTACAGCAACTTCGTCAGCTTC   840
 A  S  G  V  R  T  G  T  K  I  I  I  H  L  K  S  D  C  K  E  F  S  S  E  A  R  V  R  D  V  V  T  K  Y  S  N  F  V  S  F
CCCTTGTACTTGAATGGAAGGCGGATGAACACCTTGCAGGCCATCTGGATGATGGACCCCAAGGATGTCCGTGAGTGGCAACATGAGGAGTTCTACCGCTACGTCGCGCAGGCTCACGAC   960
 P  L  Y  L  N  G  R  R  M  N  T  L  Q  A  I  W  M  M  D  P  K  D  V  R  E  W  Q  H  E  E  F  Y  R  Y  V  A  Q  A  H  D
AAGCCCCGCTACACCCTGCACTATAAGACGGACGCACCGCTCAACATCCGCAGCATCTTCTACGTGCCCGACATGAAACCGTCCATGTTTGATGTGAGCCGGGAGCTGGGCTCCAGCGTT  1080
 K  P  R  Y  T  L  H  Y  K  T  D  A  P  L  N  I  R  S  I  F  Y  V  P  D  M  K  P  S  M  F  D  V  S  R  E  L  G  S  S  V
GCACTGTACAGCCGCAAAGTCCTCATCCAGACCAAGGCCACGGACATCCTGCCCAAGTGGCTGCGCTTCATCCGAGGTGTGGTGGACAGTGAGGAGATTCCCCTGAACCTCAGCCGGGAG  1200
 A  L  Y  S  R  K  V  L  I  Q  T  K  A  T  D  I  L  P  K  W  L  R  F  I  R  G  V  V  D  S  E  E  I  P  L  N  L  S  R  E
CTGCTGCAGGAGAGCGCACTCATCAGGAAACTCCGGGACGTTTTACAGCAGAGGCTGATCAAATTCTTCATTGACCAGAGTAAAAAAGATGCTGAGAAGTATGCAAAGTTTTTTGAAGAT  1320
 L  L  Q  E  S  A  L  I  R  K  L  R  D  V  L  Q  Q  R  L  I  K  F  F  I  D  Q  S  K  K  D  A  E  K  Y  A  K  F  F  E  D
TACGGCCTGTTCATGCGGGAGGGCATTGTGACCGCCACCGAGCAGGAGGTCAAGGAGGACATAGCAAAGCTGCTGCGCTACGAGTCCTCGGCGTCCGGGCAGCTAACCAGCCTCTCAGAA  1440
 Y  G  L  F  M  R  E  G  I  V  T  A  T  E  Q  E  V  K  E  D  I  A  K  L  L  R  Y  E  S  S  A  S  G  Q  L  T  S  L  S  E
TACGCAGCGGCACTGGGCCGGCACCCGCAACATCTACTACCTGTGCGCCCCCAACCGTCACCTGGCAGAGCACTCACCCTACTATGAGGCCATGAAGAAGAAAGACACAGAGGTTCTCTTC  1560
 Y  A  A  H  W  A  G  T  R  N  I  Y  Y  L  C  A  P  N  R  H  L  A  E  H  S  P  Y  Y  E  A  M  K  K  K  D  T  E  V  L  F
TGCTTTGAGCAGTTTGATGAGCTCACCCTGCTGCACCTTCGTGAGTTTGACAAGAAGAAGCTGATCTCTGTGGAGACGGACATAGTCGTGGATCACTACAAGGAGGAGAAGTTTGAGGAC  1680
 C  F  E  Q  F  D  E  L  T  L  L  H  L  R  E  F  D  K  K  K  L  I  S  V  E  T  D  I  V  V  D  H  Y  K  E  E  K  F  E  D
AGGTCCCCAGCCGCCGAGTGCCTATCAGAGAAGGAGACGGAGGAGCTCATGGCCTGGATGAGAAATGTGCTGGGGTCGCGTGTCACCAACGTGAAGGTGACCCTCCGACTGGACACCCAC  1800
 R  S  P  A  A  E  C  L  S  E  K  E  T  E  E  L  M  A  W  M  R  N  V  L  G  S  R  V  T  N  V  K  V  T  L  R  L  D  T  H
CCTGCCATGGTCACCGTGCTGGAGATGGGGGCTGCCCGCCACTTCCTGCGCATGCAGCAGCTGGCCAAGACCCAGGAGGAGCGCGCACAGCTCCTGCAGCCCACGCTGGAGATCAACCCC  1920
 P  A  M  V  T  V  L  E  M  G  A  A  R  H  F  L  R  M  Q  Q  L  A  K  T  Q  E  E  R  A  Q  L  L  Q  P  T  L  E  I  N  P
AGGCACGCGCTCATCAAGAAGCTGAATCAGCTGCGCGCAAGCGAGCCTGGCCTGGCTCAGCTGCTGGTGGATCAGATATACGAGAACGCCATGATTGCTGCTGGACTTGTTGATGACCCT  2040
 R  H  A  L  I  K  K  L  N  Q  L  R  A  S  E  P  G  L  A  Q  L  L  V  D  Q  I  Y  E  N  A  M  I  A  A  G  L  V  D  D  P
AGGGCCATGGTGGGCCGCTTGAATGAGCTGCTTGTCAAGGCCCTGGAGCGACACTGA                                                                2189
 R  A  M  V  G  R  L  N  E  L  L  V  K  A  L  E  R  H  .
CAGCCAGGGGGCCAGAAGGACTGACACCACAGATGACAGCCCCACCTCCTTGAGCTTTATTTACCTAAAGGTATTTCTTAACCCGAAAAAAA
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FIG. 2. Nucleotide sequence of the longest C41 cDNA and encoded amino acid sequence of the longest open reading frame. The open reading frame, which includes the most 5′ end of the DNA sequence, may start with an initiation codon other than the usual AUG (4), with the first amino acid tentatively corresponding to the 50th codon (TTG). The 3′ end of the untranslated sequence is very short and contains a potential polyadenylation addition signal.

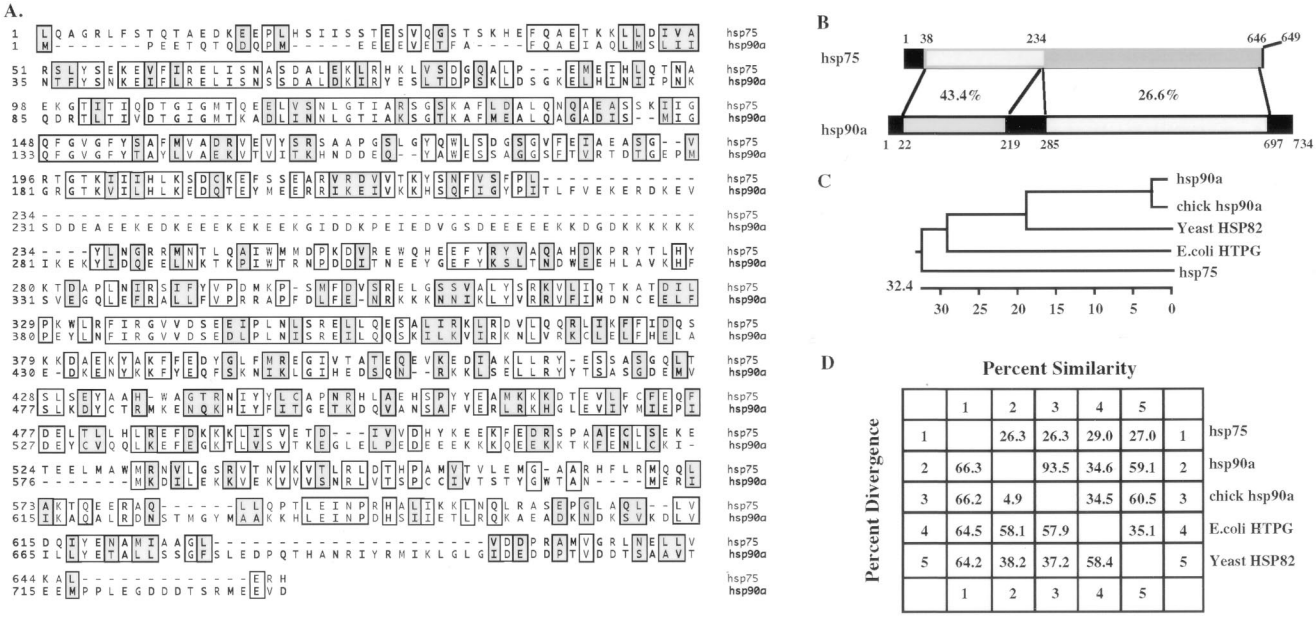


FIG. 3. The protein encoded by C41 has sequences homologous to those of the hsp90 family. (A) Sequence comparison between C41 and hsp90a. The black boxes show identical primary structures, and the open boxes show amino acids with similar chemical properties. Two stretches of sequences in hsp90a were not found in the protein (hsp75) encoded by the C41 cDNA. (B) Diagram of homology between hsp75 and hsp90. (C and D) Evolutionary tree (C) and similarity between hsp75 and other members of the hsp90 family (D), obtained by the Clustal method provided by DNASTAR.

homology with amino acids 285 to 697 in hsp90a. The N-terminal regions, the middle regions, and the C-terminal regions of the two proteins are distinct and differ greatly. A detailed comparison of members of this family of proteins is shown in Fig. 3A and B. Computer-assisted analysis of the phylogenic tree of the putative C41 gene product and members of the hsp90 family (Fig. 3C and D) suggests that hsp75 is a distant relative of hsp90. Little (13.1%) or no sequence homology with hsp70 was found in the newly identified protein when primary sequences were compared by using the same computer program (DNASTAR).

Anti-C41 antibodies detect a ubiquitously expressed 75-kDa protein in mammalian cells. To identify the cellular protein encoded by the C41 gene, two plasmids, GST-C41 (encoding amino acids 428 to 649) and GST-C41N (encoding amino acids −24 to 252), were constructed for expressing GST-C41 fusion proteins in bacteria. The fusion proteins were then used as antigens to immunize mice and generate polyclonal antibodies. To identify the authentic C41 protein in mammalian cells, [^{35}S]methionine-labeled CV-1 cell lysates were prepared and used for immunoprecipitation either with anti-C41 (amino acids 428 to 649) antibody (for simplicity, this antibody is hereafter called anti-C41) or with preimmune serum. Immunoprecipitates were separated by SDS-PAGE and autoradiographed (Fig. 4A). Anti-C41 recognized a 75 kDa protein lacking in the immunoprecipitates of the same cells brought down by preimmune serum (compare lanes 1 and 2 in Fig. 4A). Depletion of the antibody by preincubation with GST-C41 fusion protein abolished the specific immunoprecipitation, while incubation with GST alone had no effect (Fig. 4A, lanes 4 and 3). Specificity for the 75-kDa protein was further confirmed by reimmunoprecipitation of the proteins obtained in the first immunoprecipitate, following their recovery by denaturation (Fig. 4A, lane 5). Similar results were also obtained with a different polyclonal antibody, anti-C41N (data not shown). These results suggest that the 75-kDa cellular protein is the gene product of C41. We have named this product hsp75 on the basis of its apparent molecular mass and its homology with hsp90 family members.

The specificity of anti-C41 antibodies and the size of the protein encoded by the longest cDNA were confirmed by directly comparing the in vitro-transcribed and -translated C41 gene product with the cellular hsp75 immunoprecipitated from CV1 cells. As shown in Fig. 4B, the second species of the translated products from the full-length cDNA has a mobility in SDS-PAGE similar to that of the cellular hsp75. This migration pattern in turn suggests that the cloned 2.2-kb cDNA encodes a full-length protein and that the codon at the +50 position may be the initiation site for the cellular protein. Similar, nonstandard codons are used to initiate the transcription of several other genes (4). However, our current data do not allow us to determine the initiation codon unambiguously. The hsp75 initiation site was tentatively assigned to the 50th codon.

We then used the polyclonal anti-C41 antibody to perform Western blotting analysis of cell lysates prepared from different organs of adult mice. As shown in Fig. 4C, hsp75 was detected in all cell lysates. The protein is therefore ubiquitously expressed in many different kinds of mammalian cells.

hsp75 uses an LxCxE motif to bind to Rb in the T-antigen-binding domains. To map the sequences in Rb needed for binding to hsp75, a series of mutants with mutations in the T-binding domain of Rb were created and subcloned into pAS1, which contains the yeast Gal4 transactivation domain. These mutants were used to cotransform *S. cerevisiae* Y153 with the originally isolated C41 clone, and the resulting colonies were analyzed for β-galactosidase activity (Fig. 5). hsp75 fails to bind to those deletion mutants with mutations that affect either the A or B subdomain of the T-antigen-binding domain of Rb (26, 28). A similar loss of binding to Rb was also observed when SV40 T antigen was used instead of hsp75 in an identical assay. Thus, a largely intact T-binding domain in Rb

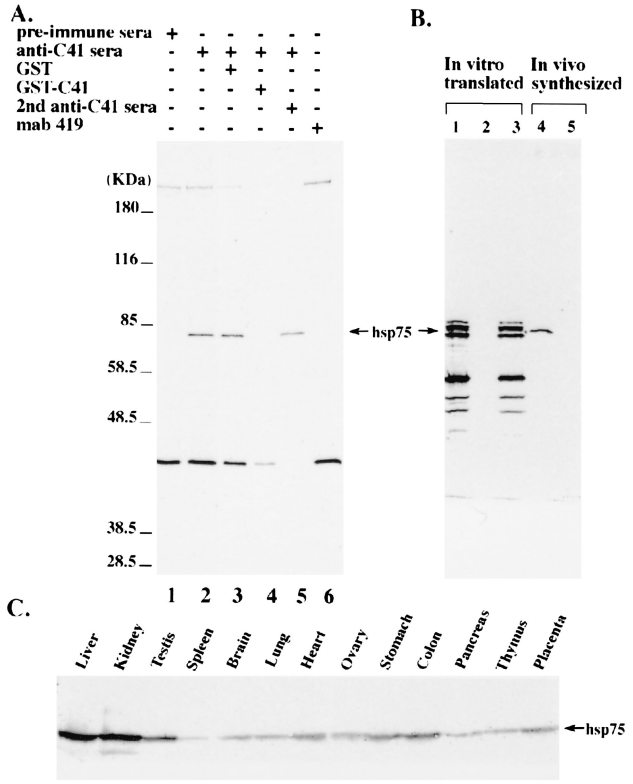
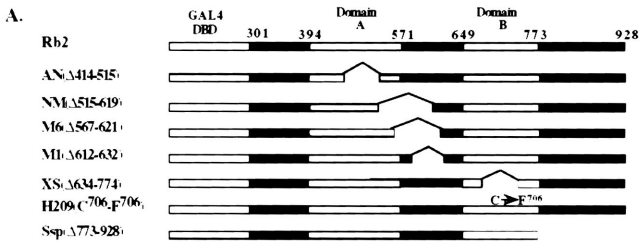


FIG. 4. Characterization of the protein encoded by C41. (A) Identification of a cellular protein by anti-C41 antibodies. Lysates of CV1 cells labeled with [^{35}S]methionine were immunoprecipitated with preimmune serum (lane 1), anti-C41 alone (lane 2), anti-C41 after treatment with GST (lane 3), or anti-C41 after treatment with the original GST-C41 antigen (lane 4). Lane 5, double immunoprecipitation with anti-C41 to remove coimmunoprecipitating proteins. A cellular protein with an apparent molecular mass of about 75 kDa is specifically recognized by the antibody. Monoclonal antibody (mab) 419 (lane 6), which recognizes SV40 T antigen, was used here as an additional antibody control to identify nonspecific protein bands in the gel. (B) Comparison between the in vitro-transcribed and -translated protein from C41 cDNA and the cellular protein identified as described above. Lane 1, immunoprecipitation and developing antibody was C41 serum; lane 2, preimmune serum; lane 3, immune serum preabsorbed with GST; lane 4, anti-C41 serum; lane 5, preimmune serum. The second most slowly migrating band from the in vitro-translated proteins assumed a position similar to that of cellular hsp75 and thereby suggested that the isolated cDNA does indeed encode full-length protein. The protein corresponding to the second band in lanes 1 and 3 probably initiates from either codon 50 or 55. (C) hsp75 is ubiquitously expressed in mouse tissues. Lysates prepared from various mouse organs were directly analyzed by Western blotting with anti-C41 antibodies as a probe. All tissues analyzed expressed hsp75.



B.

DNA Binding Domain Fusion	Color-c41	Activation Domain Fusion (units of β-galactosidase) c41	color-T
Rb2	blue	327 ± 27.5	blue
AN	white	15.4 ± 1.3	white
NM	white	9.45 ± 3.15	white
M6	white	3.7 ± 1.3	white
M1	blue	271.5 ± 69.5	blue
XS	white	5.6 ± 0.7	white
H209	white	0.16 ± 0.16	white
Ssp	white	1.55 ± 0.99	white

FIG. 5. hsp75 binds to the SV40 T-antigen-binding domain of Rb. (A) A panel of Rb mutants fused to the DNA-binding domain of Gal4 in the pAS1 vector. (B) The pSE-C41(Gal4-hsp75) fusion construct was derived from the C41 cDNA fragment of the original screen in the yeast two-hybrid system. Transformation of yeast strain Y153 with the pAS-RB series and with pSE-C41 or pJBTB-T (SV40 T antigen) was performed as previously described (16). β-Galactosidase activities were recorded either by colorimetry or by quantitation with CPRG. C41 binds to Rb in a region similar to that used by SV40 T antigen.

is required for the interaction between hsp75 and Rb. The same domain in Rb is used to bind to many known proteins such as E2F-1, E1A, and T antigen (for a review, see reference 8).

An analysis of the primary structure of hsp75 identified a perfect LxCxE sequence located at amino acids 470 to 474. Similar LxCxE motifs are used in the binding of several other proteins to Rb (8). The LxCxE sequence in hsp75 is located within a region highly conserved in other members of the hsp90 family. In the other members, however, the cysteine residue is replaced with methionine. To determine whether LxCxE is critical for the binding to Rb, a single point mutation changing cysteine to methionine was made in the original C41 clone, to create C41C472-M. An assay similar to the one described above was then used to measure the binding of C41C472-M to Rb. The binding activity of the point-mutated C41 was orders of magnitude weaker than that of the wild-type C41 (Fig. 6A). This lack of binding was not merely due to the failure of expression of C41C472-M protein in *S. cerevisiae*; the expression levels of wild-type C41 and C41C472-M in yeast cells were comparable (Fig. 6B). The lack of binding of an hsp75 with a single point mutation within its LxCxE sequence suggests that the LxCxE motif in hsp75 is critical for binding to Rb. Other members of the hsp90 family of proteins may fail to bind to Rb because they contain methionine instead of cysteine in this crucial domain.

To demonstrate that Rb and hsp75 interact specifically in vitro, both the wild-type and mutant full-length hsp75 cDNAs were used for constructing GST-fusion proteins. These two fusion proteins were then used to examine the abilities of the wild-type and mutant hsp75 to bind to endogenous cellular Rb and to determine the form of Rb with which they interact. As shown in Fig. 6C, wild-type hsp75 specifically bound to hypophosphorylated Rb, as did the positive control, T antigen. The mutant (C472-M) failed to bind any detectable Rb, as did the negative control, GST alone. Taken together, these results demonstrate that the binding of hsp75 with cellular Rb is specific.

hsp75 localizes in cytoplasm. To examine the potential ability of hsp75 to interact with Rb in mammalian cells, the cellular localization of the hsp75 was determined by immunostaining and cellular fractionation. Normal monkey kidney (CV1) cells were fixed, prepared, and stained with the same anti-C41 antibody used in immunoprecipitation experiments. Subsequent visualization via indirect immunofluorescence of a fluorescein isothiocyanate-tagged, anti-mouse IgG secondary antibody demonstrated that hsp75 distributes to a specific cytoplasmic region immediately surrounding the nucleus (Fig. 7A, panels c and d). This immunostaining pattern is specific for hsp75, since competition with the GST-C41 fusion protein abolishes the staining while competition with GST alone does not (Fig. 7A, panels e to h). Cell fractionation studies were consistent with

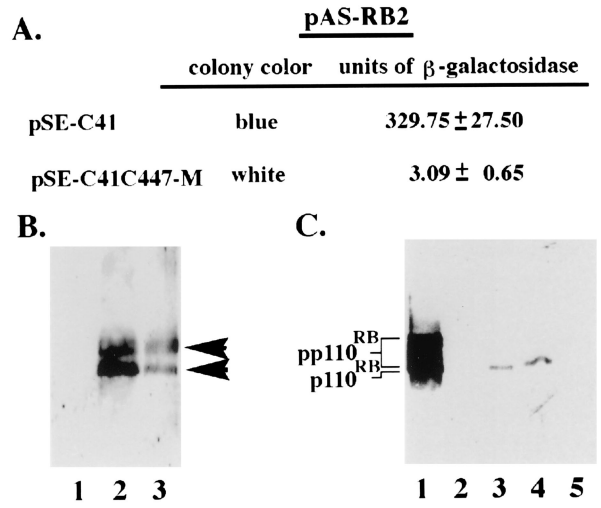


FIG. 6. The amino acid sequence LxCxE in hsp75 is critical for binding to Rb. (A) A mutant hsp75 in which cysteine 472 is changed to methionine fails to bind to Rb. The abilities of the wild-type and mutant C41 to interact with the wild-type Rb (pAS-RB2) were assayed as described elsewhere (16). β-Galactosidase activity was reduced more than 100-fold with the mutant construct; this indicated that the mutated hsp75 protein failed to interact with Rb. (B) Expression of wild-type hsp75 and the C472-M mutant in yeast cells. The total proteins from yeast strain Y153 alone (lane 1) and from the same strain transformed by pSE-C41 (lane 2) and by pSE-C41C472-M (lane 3) were analyzed by Western blotting with anti-Gal-TA antibodies. The expression levels of the wild-type hsp75 and the mutant protein were comparable. (C) C41 binds the hypophosphorylated form of Rb by using an LxCxE motif. GST fusion proteins with either the C472-M mutant hsp75 (lane 5) or the wild-type hsp75 (lane 4) were expressed in bacteria and purified with glutathione-agarose beads. The GST-T fusion protein (lane 3) and GST protein alone (lane 2) served as positive and negative controls, respectively, for Rb binding. Cell lysates containing Rb in different states of phosphorylation were either immunoprecipitated by the anti-Rb 11D7 monoclonal antibody (lane 1) or incubated with the GST-fusion protein bound to agarose beads. The immunoprecipitates or the proteins bound to these beads were then analyzed by Western blotting with the 11D7 monoclonal antibody as a probe.

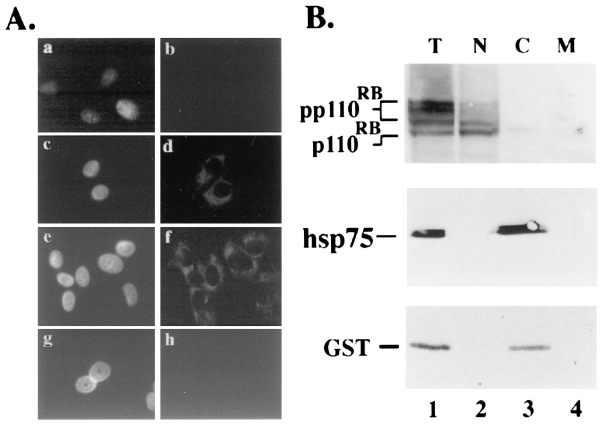


FIG. 7. Cellular localization of hsp75 by immunostaining and cell fractionation. (A) CV1 cells were stained with DAPI (a, c, e, and g) to locate the nuclei and simultaneously reacted with either preimmune serum (b) or anti-C41 antibody (d, f, and h). In panels f and h, the anti-C41 antibodies were preabsorbed with either GST protein (f) or GST-C41 (h) before staining. (B) Cell fractionation. Total CV1 cells (T) were fractionated into nuclear (N), cytoplasmic (C), and membrane (M) components, and aliquots of the lysates were analyzed directly by SDS-PAGE and Western blotting with anti-Rb or anti-C41. GST was isolated by using glutathione-beads, separated by SDS-PAGE, and stained with Coomassie blue. Rb localizes in the nuclei, while hsp75 localizes to the cytoplasm, as does the positive control, GST (lane 3).

the immunostaining staining data: hsp75 is detected mainly in the cytoplasm of fractionated cells (Fig. 7B, lane 3). Rb and cellular GST (Fig. 7B) served as internal controls for the nuclear and cytoplasmic fractions, respectively (11).

hsp75 coimmunoprecipitates with p110^{RB} during M phase. Rb is a nuclear protein, but hsp75 is not. The interaction between these two proteins must therefore be limited to certain specific time points during cell cycle progression or to special conditions when one or the other of the proteins moves from its usual subcellular compartment. To determine whether hsp75 and Rb could interact in mammalian cells, coimmunoprecipitation experiments were conducted. Simple coimmunoprecipitation experiments with either anti-Rb or anti-C41 antibodies, however, failed consistently to detect any complexes between these two proteins in normal, rapidly growing cells (Fig. 8A, lanes 1 and 7). If the interaction takes place only in a limited phase of cell cycle, however, such a failure to detect interaction in bulk populations of cells should not be unexpected. To overcome the difficulty with bulk populations of cells, we analyzed cell lysates from synchronized T24 cells, a human bladder carcinoma cell line with excellent cell cycle synchronization properties (9), at various time points during cell cycle progression. In these cells, hsp75 was found to coimmunoprecipitate with Rb, but only in M phase (Fig. 8A, lanes 6 and 12). During M phase, the nuclear envelope breaks down, and Rb distributes uniformly throughout the cytoplasm in a pattern that physically overlaps that of hsp75, as shown by double immunostaining with antibodies to both proteins (Fig. 8B). During mitosis, Rb and hsp75 may therefore have the opportunity to interact. This observation is significant, since the hyperphosphorylated form of p110^{RB} undergoes dephosphorylation in M phase (9, 38). At this stage in its modification, hypophosphorylated Rb may need to be chaperoned to facilitate its proper folding, and hsp75 could provide such a function. Thus, the Rb-hsp75 interaction may have biological significance in cells.

Rb and hsp75 interact in cells after heat shock. If the hypothesis that hsp75 is a molecular chaperone for Rb is true,

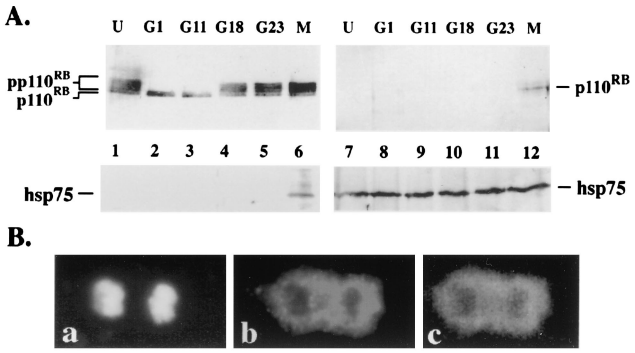


FIG. 8. hsp75 binds to Rb specifically during M phase. (A) Unsynchronized T24 human bladder carcinoma cells (U) were arrested at G_0 or G_1 by contact inhibition, and released by plating at low density for various time periods (G1 to G23, indicating hours after release from G_0-G_1 arrest). Cells in mitosis (M) were obtained after release for 24 h followed by treatment with nocodazole. About 5×10^6 cells for each time point were lysed and immunoprecipitated by either anti-Rb 11D7 (lanes 1 to 6) or anti-C41 (lanes 7 to 12) antibodies. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with either anti-Rb (upper panels) or anti-C41 (lower panels) antibodies. hsp75 coimmunoprecipitated with Rb only in M phase. (B) Colocalization of Rb and hsp75 in T24 cells arrested in M phase. (a) DAPI staining to show the condensed chromosomes in M phase. (b) Indirect immunostaining with rabbit anti-Rb antibody 0.47 followed by Texas Red-conjugated goat anti-rabbit IgG secondary antibody. (c) Immunostaining with mouse anti-C41 antibodies followed by FITC-conjugated goat anti-mouse IgG. The distributions of Rb and hsp75 in M-phase cells overlap.

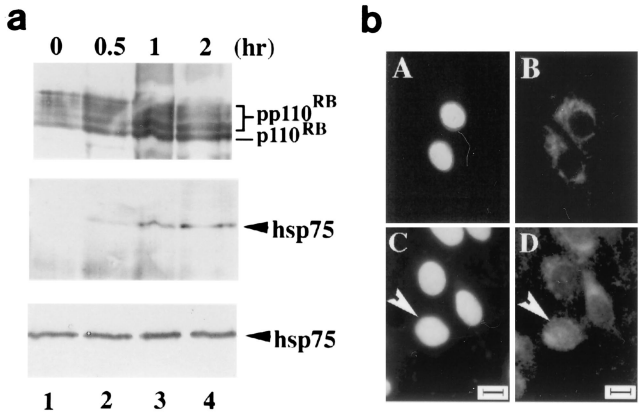


FIG. 9. hsp75 binds Rb in cells exposed to heat-shock. (a) Unsynchronized, fast-growing CV1 cells (about 5×10^6) were heat shocked at 42°C for various time periods as indicated. The cells were lysed, and aliquots of lysates were directly analyzed by SDS-PAGE and immunoblotting with anti-C41 antibody (bottom panel). The remaining lysates were immunoprecipitated with an anti-Rb antibody, 11D7 (upper and middle panels). The immunoprecipitates were then analyzed by immunoblotting with the same anti-Rb and anti-C41 antibodies, respectively. hsp75 was coimmunoprecipitated by the anti-Rb antibody after less than 1 h of heat shock. (b) hsp75 moves into the nucleus when cells are heat shocked. Micrographs show staining of CV1 cells with DAPI (A and C) and anti-C41 (B and D) before (A and B) and after (C and D) heat shock for 1 h. Bars, 20 μm.

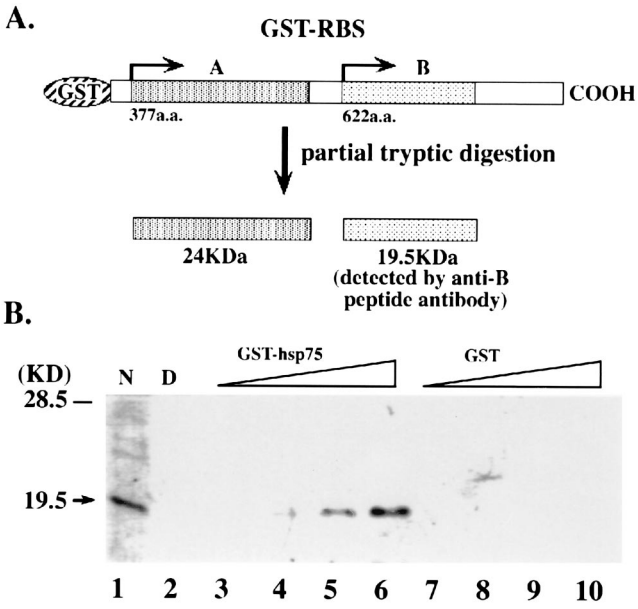


FIG. 10. hsp75 chaperones denatured Rb. (A) Diagram of the strategy employed in the experiment to detect fragments of Rb in their native conformation. Partial trypsin digestion followed by Western blotting with domain-specific antibodies recognizes individual Rb domains, as previously described (23). a.a., amino acids. (B) Blot analysis with anti-B peptide antibody detected products from either native GST-RBS (N) or denatured GST-RBS (D) digested with trypsin. Either GST-C41FL (0.1 to 1.0 μg) (lanes 3 to 6) or equivalent amounts of GST alone (lanes 7 to 10) were added to the denatured Rb before trypsin digestion. Detection of the intact B domain of Rb in lanes 4, 5, and 6 indicates that hsp75 refolds denatured Rb; GST alone has no such refolding ability.

additional time windows or special cellular conditions may exist to allow the interaction required for chaperoning. To mimic a logical and pertinent special circumstance, we examined cells after heat shock. In lysates from cells exposed to temperatures of 42°C for 30 min or longer, hsp75 could be detected in immunocomplexes precipitated by anti-Rb antibodies (Fig. 9A, lanes 3 and 4). Other heat shock proteins, such as hsp70 and hsp90, have no significant binding to Rb (data not shown). While hsp70 was easily aggregated and immunoprecipitated by several control antibodies before or after heat treatment, hsp75 did not have this property. Indirect immunofluorescence with anti-Rb and anti-C41 primary antibodies was also used to monitor the effects of heat shock on intact cells. As shown in Fig. 9B, heat shock caused translocation of hsp75 from a perinuclear, cytoplasmic location to the nucleus in a proportion of the cells. The coimmunoprecipitation studies and relocalization as determined by indirect immunofluorescence suggest that Rb and hsp75 can interact not only during M phase but also after cells encounter the stress of heat shock. Such a special circumstance may require refolding of Rb after it is partially denatured, and hsp75 may then act as a specific chaperone for Rb, as it may during M phase.

hsp75 refolds denatured Rb to its native conformation in vitro. The ability of hsp75 to chaperone Rb was tested in vitro. Purified GST-RBS (a fusion protein of GST with $p56^{RB}$), which contains the two globular subdomains (A and B) of the T-antigen-binding domain, was used as the substrate in these experiments. As previously described (23), when $p56^{RB}$ or GST-RBS is treated with trypsin for limited time periods, two predominant polypeptides, A and B, are generated (Fig. 10A and Fig. 10B, lane 1). After GST-RBS was denatured with 6 M guanidine-HCl, the A and B fragments could not be detected following treatment with trypsin (Fig. 10B, lane 2). This failure of specific cleavage is presumably due to loss of the three-dimensional conformation that normally protects the protein from more extensive proteolytic digestion. Upon addition of the GST-hsp75 fusion protein to the denatured GST-RBS, however, the A and B fragments were regenerated (Fig. 10B, lanes 4 to 6). Addition of GST protein as control failed to facilitate such refolding (Fig. 10B, lanes 7 to 10). These results suggest that hsp75 can indeed chaperone denatured Rb.

DISCUSSION

The discovery of an interaction between a heat shock protein in the hsp90 family and Rb points to a heretofore unknown mechanism by which Rb may be regulated in response to changing conditions. Phosphorylation and dephosphorylation, although clearly important, may not be the only modifications that Rb undergoes during cell cycle progression and in response to extracellular stimuli. We have shown that hsp75 can facilitate the proper folding of Rb in vitro required for specific enzymatic digestion and that it has the potential to interact with Rb in the cell, specifically during mitosis and after the stress of heat shock. Although determination of changes in the conformation of Rb at various stages of the cell cycle is not feasible, these special circumstances under which hsp75 and Rb interact are characterized by the potential need for Rb to refold or renature. At the end of mitosis, a substantial pool of the total cellular Rb has just been dephosphorylated and therefore may have assumed a different three-dimensional conformation prior to the next G_1 phase (9, 38). It is plausible to assume that dephosphorylation of Rb results in conformational changes that require refolding, and it is possible that hsp75 functions as a chaperone for Rb at this time point in cycling cells.

The other special condition under which Rb and hsp75 have the opportunity to interact functionally in vivo is after cellular stress. We have shown that after the specific stress of heat shock, hsp75 coimmunoprecipitates with Rb. The detailed mechanism by which Rb is altered by hsp75 remains to be

shown, but it is unlikely that the interaction between these two proteins is due primarily to the increase of hsp75 expression after the stress of excessive temperature. Our preliminary results indicate that the expression of hsp75 is not induced by heat shock, as is expression of other heat shock proteins (43). Heat stress appears not to increase hsp75 expression but to shuttle hsp75 from its usual perinuclear position into the nucleus, where it can interact with Rb. Heat shock could modify hsp75, change its conformation by phosphorylation or other means, expose a masked nuclear targeting signal, or cause the protein to associate as a passenger with another nuclear protein (31). Such modifications are consistent with the observation that hsp75 begins to enter the nucleus and associate with Rb only 30 min after heat stress. It will be important in the future to reveal the signal cascades resulting in the shuttling of hsp75 from the cytoplasm to the nucleus. While we were characterizing hsp75 and its interaction with Rb, a clone very similar to our C41, encoding a protein named TRAP-1, was isolated by its interaction with the type 1 tumor necrosis factor receptor (52). Besides the difference in cDNA length, the amino acid sequences in the respective N- and C-terminal regions are quite divergent.

hsp75 has significant homology with other members of the hsp90 family (Fig. 3), but it is unique among them in containing an LxCxE motif, a hallmark of many proteins that associate with Rb through the SV40 T-antigen-binding domain (8). The region immediately surrounding this LxCxE motif in hsp75 is especially conserved among hsp90 proteins, but the cysteine is replaced by methionine in all members of the family except the hsp75 reported here. Moreover, when hsp75 is mutated with a single amino acid substitution (Cys-472→Met) that makes it more like other known members of the hsp90 family, it loses its ability to bind to Rb (Fig. 6). Consistent with this observation, neither hsp90 nor hsp70 binds to Rb after heat treatment. Finally, GST-hsp75, but not GST alone, can restore the native conformation of Rb with sufficient fidelity to allow proteolytic cleavage into predictable globular components (Fig. 10). Many cellular proteins have been shown to bind to Rb. Whether binding and chaperone activities are equivalent is unknown at present. However, other Rb-associated proteins containing LxCxE motifs identical to that of hsp75, such as SV40 large T antigen and adenovirus E1a, or containing E2F motifs, such as E2F family proteins, C/EBP family proteins, or H-nuc (cdc27), and others (10), have not been shown to have chaperone activity. Further experiments are needed to clarify whether the specific binding is essential for the chaperone activity of hsp75.

Nonetheless, hsp75 indeed does appear to have chaperone activity for Rb in vitro. At present we do not know whether this activity of hsp75 is specific for Rb or whether other heat shock proteins can also chaperone Rb. It has recently been proposed that a 73-kDa heat shock protein cognate (73hsc), a member of the hsp70 family, interacts with $p110^{RB}$ through its N-terminal region (29, 42). 73hsc was also proposed to be a specific molecular chaperone for Rb. The interaction between 73hsc and Rb, however, was demonstrated only by in vitro binding assays. It remains to be shown whether Rb and 73hsc can interact in cells as Rb and hsp75 do.

The precise conformational changes induced by hsp75 that allow Rb to refold after heat shock and other specific stresses are unknown and will be difficult to determine (17, 22). Likewise, and for many of the same reasons, the mechanism by which hsp75 chaperones Rb will be a challenging issue to resolve, as it has been for other proteins (53, 55). The T-antigen-binding domains of Rb are important in binding several proteins. The LxCxE motif is common to many of these proteins, including hsp75, cyclin D, E1A, and others (8). Another motif, Y(X7)E(X3)DLF, is found in both E2F and C/EBP proteins. Proteins such as H-nuc, C5, and others have no clear signature, and their binding motifs are yet to be determined. Rb can actually bind to similar motifs in two different proteins and yet result in opposite consequences. For example, Rb binds to E2F-1 and inactivates it but activates the function of NF-IL6 by binding to a similar region (7). These results suggest that intrinsic differences between the interacting proteins in regions other than those used directly for binding may be responsible for the opposing effects. For the interaction of hsp75 and Rb, many unanswered questions remain. How does hsp75 promote the process of refolding denatured Rb? Does the LxCxE motif of hsp75 serve as a mold for Rb to refold, or do other regions of hsp75 refold the denatured Rb until it can bind to the LxCxE motif? These molecular details await further investigation.

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