IDENTIFICATION OF A NOVEL REGION OF pRb-MEDIATING PROTEIN INTERACTION*

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Fifty-kDa heat shock cognate protein interacts directly with the N-terminal region of the retinoblastoma gene product pRb

70-kDa heat shock cognate protein interacts directly with the N-terminal region of the retinoblastoma gene product pRb

Retinoblastoma protein (pRb) functions as a tumor suppressor, and certain proteins are known to bind to pRb in the C-terminal region. Although the N-terminal region of pRb may also mediate interaction with some proteins, no such protein has been identified yet. We demonstrated previously that the in vivo protein association between pRb and 73-kDa heat shock cognate protein (hsc73) in certain human tumor cell lines. In this report we analyzed the interaction between these two proteins in vitro. Our data showed that hsc73 interacts with the novel N-terminal region of pRb; that is, pRb binds directly to hsc73 and dissociates from hsc73 in an ATP-dependent manner. By using deletion mutants of cDNA encoding pRb, the hsc73 binding site of pRb was determined to be located in the region (residues 301-372) outside the so-called A pocket (residues 373-579) of this tumor suppressor protein. This finding was compatible with the fact that the adenovirus E1A oncprotein, which is known to bind to the E2F binding pocket region of pRb, could not compete with hsc73 for the binding. Furthermore, phosphorylation of pRb by cyclin-dependent kinase inhibited the binding of pRb to hsc73. These data suggest that hsc73 may act exclusively as the molecular chaperone for nonphosphorylated pRb. As a result, hsc73 may function as a molecular stabilizer of nonphosphorylated pRb.

A 110-kDa retinoblastoma gene product (pRb) is a nuclear phosphoprotein that operates as a cell cycle regulator and as a major target of the oncoproteins of several DNA tumor viruses such as adenovirus E1A and papilloma virus E7 (1-6). Only the nonphosphorylated or the hypophosphorylated form of pRb, which predominates during the G1 phase in the cell cycle, can bind to transcriptional factor E2F (7) and inhibit the exit from G1 (8). pRb is phosphorylated by cyclin-dependent kinases in the late G1 phase (10, 11), resulting in the dissociation of the E2F-pRb complex and the activation of E2F-dependent promoters. The viral oncopro- teins E1A can bind to the E2F binding region of pRb, thus abrogating the suppressive function of pRb. The E1A or SV40 large T binding region of pRb has been mapped to the two nonconsecutive segments, referred to as the A pocket (residues 373-579) and B pocket (residues 640-771) (3, 12, 13).

The C-terminal region of pRb, downstream from the A/B pockets, is also known to be a site for mediating protein-protein interactions (14). One of the proteins that can bind to this region (residues 768-928) in pRb is a nuclear c-Abl tyrosine kinase (15). Although the c-Abl-pRb interaction is not affected by viral oncoproteins, it is disrupted by the phosphorylation of pRb during cell cycle progression. Recently it was suggested that the N-terminal region of pRb may also interact with some proteins, although no such protein has been definitively identified yet.

We reported previously that pRb could be associated in vivo with 73-kDa heat shock cognate protein (hsc73) in TYK-nu human ovarian carcinoma cells and in HeLa cervical carcinoma cells and that this complex could be dissociated in the presence of ATP (16). We further analyzed the molecular interaction and mapped the hsc73 binding region of pRb. In this report we showed that hsc73 can bind directly to an N-terminal region outside the pockets (residues 301-372 adjacent to the N-terminal boundary of the A pocket) and that phosphorylation of pRb inhibits this association in vitro and perhaps in vivo.

MATERIALS AND METHODS

Expression and Purification of Intact pRb Using the Baculovirus System—A plasmid p4.95BT (from Dr. T. P. Dryja, Harvard Medical School) was digested with BsaHI and HindIII. The resultant 4.1-kilobase fragment, which contained the entire pRb coding region, was purified from an agarose gel. The termini of the fragment were blunted with T4 DNA polymerase and then ligated to the Smal site of the pACYC184 vector (17). Transformation was done as described previously (17).

Purification of pRb was done by column chromatography using phosphocellulose (stepwise elution with 0.1, 0.25, 0.5, 0.75, and 1.0 M NaCl), heparin-Sepharose (stepwise elution with 0.1, 0.25, 0.5, and 1.0 M NaCl), and Q-Sepharose (stepwise elution with 0.05, 0.1, 0.3, 0.5, and 1.0 M NaCl). Fractions containing pRb were determined by staining with Coomassie Brilliant Blue or Western blotting as described in a previous paper (2). Purified Proteins and Antibodies—Production of recombinant adenovirus E1A protein was performed as described previously (18). Bovine brain 70-kDa heat shock protein, consisting mainly of hsc73 and human 90-kDa heat shock protein, was purchased from StressGen (Victoria, B.C., Canada).

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BC, Canada). Mycobacterial 65-kDa heat shock protein (m65hsp) was purified as described previously (19). Anti-pRb mAb, such as MH-Rb-02P (20) (mouse IgG1), recognizing an epitope of pRb amino acids 300–380, G99–549 (21) (mouse IgG1), recognizing pRb 514–610, and G99–2005 (21) (mouse IgG1), recognizing pRb 1–240, anti-human hsc73/pF72 mAb 3a3 (mouse IgG1), and antiadenovirus E1A mAb M73 (mouse IgG2a) were purchased from Pharmacia (Uppsala, Bedford, MA). After blocking nonspecific binding of proteins to the membrane with 10% skim milk at room temperature for 2 h, mAb at appropriate dilutions were reacted at room temperature for 1.5 h. The membranes were washed with 0.1% Tween 20 and phosphate-buffered saline and were reacted for 30 min with peroxidase-conjugated goat anti-mouse IgG–1GM (H+L) diluted at 1:1,000. The band detection was performed by developing for 15–30 s with ECL detection reagents (RPN2105, Amersham Corp.) according to the manufacturer’s instructions.

RESULTS

Association of hsc73 and pRb in Vitro and Dissociation by ATP—To determine whether hsc73 interacts directly with pRb we tested the ability of purified hsc73 to bind to purified pRb in vitro. Intact pRb was produced using the baculovirus system and purified by several steps of column chromatography. As shown in Fig. 1A (lane 1), staining of with Coomassie Brilliant Blue demonstrated only one band corresponding to approximately 110 kDa in molecular size, indicating a high purity (>98.0%) of pRb preparation.

To confirm further the direct association of these proteins, hsc73 was precipitated with anti-hsc73 mAb and analyzed by immunoblotting with anti-pRb mAb or anti-hsc73 mAb. As shown in Fig. 1B, anti-pRb mAb precipitated pRb having a molecular mass of 110 kDa (lane 1). Anti-hsc73 MAb (3a3) detected a 73-kDa protein in the pRb immunoprecipitates (lane 4). Thus, purified hsc73 appeared to co-precipitate with purified pRb.

Since the in vivo hsc73-pRb complex recovered from cell lysates had been proven previously to be dissociated in the presence of ATP (16, 26), we tested the ATP-dependent dissociation of the complex formed in vitro with purified proteins. An equal molar ratio of pRb and hsc73 was mixed, followed by the addition of ATP, ADP, or ATP-γ-S at 37°C. The cell lysates were reacted with 3a3 mAb, and the immunoprecipitates were Western blotted to the Immobilon membranes (Millipore, Bedford, MA). After blocking nonspecific binding of proteins to the membrane with 10% skim milk at room temperature for 2 h, mAb at appropriate dilutions were reacted at room temperature for 1.5 h. The membranes were washed with 0.1% Tween 20 and phosphate-buffered saline and were reacted for 30 min with peroxidase-conjugated goat anti-mouse IgG–1GM (H+L) diluted at 1:1,000. The band detection was performed by developing for 15–30 s with ECL detection reagents (RPN2105, Amersham Corp.) according to the manufacturer’s instructions.

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that heat shock protein families act as molecular chaperones for various proteins, other families of hsp may interact with pRb.

To assess the specificity of the protein interaction between hsc73 and pRb, an equal molar ratio of purified m65hsp and purified hsp90 was used for the immunoprecipitation experiment. Specific mAbs, anti-m65hsp mAb (B97) and anti-hsp90 mAb (386), were used to detect these hsps in immunoblotting. These mAbs were shown to detect the specific hsps having compatible molecular masses in the immunoblotting (Fig. 3, lanes 8–10). Using the condition in which hsc73 (lane 5) was coprecipitated with pRb, neither m65hsp (lane 6) nor hsp90 (lane 7) was detected in pRb immunoprecipitates. These data suggest that the protein interaction of hsc73 and pRb is specific among hsp families.

Mapping of the hsc73 Binding Site in pRb—To determine the hsc73 binding site in pRb, we assessed the ability of purified pRb to bind a series of GST fusion proteins containing different deletion mutants of pRb. The five different deletion mutants prepared contained the N-terminal 300 amino acids (GST-del Rb1–300), 514 amino acids (GST-del Rb1–514), and the C-terminal 555 amino acids (GST-del Rb1–602), 213 amino acids (GST-del Rb1–300), 514 amino acids (GST-del Rb1–514), 602 amino acids (GST-del Rb1–602), and 80-kDa band (Fig. 3, lane 5) of GST-del Rb1–300, may overlap non-specific bands with Mh-Rb-02P mAb seen in lane 4A, arrow in lane 5, respectively. These data suggest that hsc73 may interact with a region containing the N-terminal non-pocket region of 72 amino acids (residues 301–372) adjacent to the N-terminal boundary of the A pocket in pRb as summarized in Fig. 4C.

The possibility of hsc73 binding to the pocket regions was excluded by way of a competition experiment. Viral oncoprotein...
E1A is known to bind to so-called A/B pockets of pRb (Fig. 4C) (3, 4). We tested whether purified E1A protein could compete with hsc73 for binding to pRb in vitro. pRb was immunoprecipitated from the mixture of equal molar amounts of purified hsc73 and purified pRb in the presence or absence of the same molar amounts of purified E1A protein, then subjected to SDS-PAGE and analyzed by immunoblotting with anti-hsc73 mAb. As shown in Fig. 5, pRb could form a complex with hsc73 independent of the presence of E1A protein. The amount of hsc73 coprecipitated with pRb did not differ between the absence (Fig. 5, lane 1) and presence (lane 2) of E1A. Furthermore, the amount of E1A protein coprecipitated with pRb did not differ between the presence (lane 4) and absence (lane 5) of hsc73. These data indicate that hsc73 can associate with pRb in a region distinct from the E1A binding region, suggesting the interaction of hsc73 with the nonpocket region of pRb.

Hsc73 Can Interact with Nonphosphorylated pRb but Not with Phosphorylated pRb in Vitro and in Vivo—Since all of the known pRb-binding proteins can associate only with the nonphosphorylated or hypophosphorylated form of pRb, we analyzed the phosphorylation dependence of the hsc73-pRb physical protein association. Although pRb preparations used in the current experiments appear to contain a weak endogenous kinase activity (Fig. 6A, lane 1), pRb could be phosphorylated efficiently by exogenously added cyclin-p34cdc2 kinase complex in vitro in the presence of ATP (lane 2). Hsc73 was not phosphorylated at all (lanes 3 and 4). Then, we tested the coprecipitation of hsc73 or E1A protein with pRb phosphorylated by p34cdc2 kinase in vitro (Fig. 6B, lanes 1, 4, and 7). As a nonphosphorylation control, two cases were prepared: pRb and p34cdc2 kinase without ATP (lanes 3, 6, and 9), and pRb and ATP without p34cdc2 kinase (lanes 2, 5, and 8). Samples were incubated with hsc73 or E1A protein followed by immunoprecipitation with anti-pRb mAb and immunoblotting with anti-hsc73 mAb or anti-E1A protein mAb. Both hsc73 and E1A were detected in the pRb precipitates where pRb was not phosphorylated (lanes 5, 6, 8, and 9), whereas neither hsc73 nor E1A could be detected in the sample where pRb was hyperphosphorylated (lanes 4 and 7).

We further confirmed that hsc73 could associate preferentially in vivo with the nonphosphorylated and hypophosphorylated form of pRb. Since it was shown previously that okadaic acid treatment could phosphorylate pRb in vivo (25), we treated HOS cells with this agent and obtained the phosphorylated form of pRb (Fig. 7, lane 1). In contrast, HOS cells without okadaic acid treatment showed a rather broad band corresponding to phosphorylated and nonphosphorylated pRb (lane 2). Then we made the immunoprecipitates with 3a3 mAb and analyzed by Western blotting and analyzed by immunoblotting with anti-hsc73 mAb or anti-E1A protein mAb. Both hsc73 and E1A were detected in the precipitates where pRb was not phosphorylated (lanes 5, 6, 8, and 9), whereas neither hsc73 nor E1A could be detected in the sample where pRb was hyperphosphorylated (lanes 4 and 7).

These in vitro and in vivo data indicate that hsc73 can interact with the nonphosphorylated or hypophosphorylated form but not with hyperphosphorylated form of pRb, suggesting that there might be a regulatory mechanism of the molecular interaction similar to that of other pRb-binding proteins such as viral oncproteins and transcription factors. It is speculated that hsc73 may dissociate from the pRb complex follow-
nM okadaic acid for 2 h, precipitates were immunoblotted. The lysate alone of HOS cells with 100 µM cold ATP and 10–30 ng of cyclin-p34cdc2 complex at 25 °C for 30 min containing 0.1 mM cold ATP and 10–30 µCi of [32P]ATP. Phosphorylated pRb was visualized by autoradiography of X-ray film. Panel B, hyperphosphorylated pRb (3 µg) (lanes 1, 4, and 7) and non- or hypophosphorylated pRb (3 µg) (lanes 2, 3, 5, 6, and 9) were incubated with purified hsc73 (2 µg) and E1A (2 µg) and immunoprecipitated by anti-pRb Mh-Rb-02P mAb. The immunoprecipitates were resolved by 8% SDS-PAGE and analyzed by immunoblotting with anti-pRb Mh-Rb-02P mAb (lanes 1–3), anti-hsc73 3a3 mAb (lanes 4–6), and anti-E1A M73 mAb (lanes 7–9). The bands were detected by developing for 30 s with ECL detection reagents.

**FIG. 6. Selective binding of hsc73 to non- or hypophosphorylated pRb.** Panel A, purified pRb (1 µg) or hsc73 (1 µg) was incubated with (lanes 2 and 4) or without (lanes 1 and 3) 30 ng of cyclin-p34cdc2 complex at 25 °C for 30 min containing 0.1 mM cold ATP and 10–30 µCi of [32P]ATP. Phosphorylated pRb was visualized by autoradiography of X-ray film. Panel B, hyperphosphorylated pRb (3 µg) (lanes 1, 4, and 7) and non- or hypophosphorylated pRb (3 µg) (lanes 2, 3, 5, 6, and 9) were incubated with purified hsc73 (2 µg) and E1A (2 µg) and immunoprecipitated by anti-pRb Mh-Rb-02P mAb. The immunoprecipitates were resolved by 8% SDS-PAGE and analyzed by immunoblotting with anti-pRb Mh-Rb-02P mAb (lanes 1–3), anti-hsc73 3a3 mAb (lanes 4–6), and anti-E1A M73 mAb (lanes 7–9). The bands were detected by developing for 30 s with ECL detection reagents.

**FIG. 7. In vivo physical association of nonphosphorylated pRb with hsc73.** The lysates of HOS cells were immunoprecipitated with (lane 3) or without (lane 4) anti-hsc73 3a3 mAb, and these immunoprecipitates were immunoblotted. The lysate alone of HOS cells with 100 mM okadaic acid treatment for 2 h (lane 1) or without treatment (lane 2) was also employed. The mixture of 50 µl of cell lysate alone and 50 µl of SDS sample buffer was run on 8% SDS-PAGE and immunoblotted. These blots were subsequently detected by anti-Rb Mh-Rb-02P mAb as described above. The bands were detected by developing for 30 s with ECL detection reagents. ppRb and pRb indicate a mobility in SDS-PAGE of the phosphorylated and nonphosphorylated forms of pRb, respectively.

**DISCUSSION**

pRb plays an important role in regulating the cell cycle by interacting with some nuclear proteins. The regions of pRb mediating protein interactions have been identified. The region in pRb which mediates the interaction with transcriptional factor E2F is located in the C-terminal region containing regions referred to as A/B pockets (residues 373–771) (7) and is necessary in the growth-suppressive function of pRb (8, 9). Several viral oncoproteins can bind to the pocket region and block the binding of pRb to E2F, resulting in an uncontrolled transcriptional activation and cellular transformation. Nuclear c-Abl tyrosine kinase was also shown to interact with pRb (15). Unlike viral oncoproteins, the c-Abl binding region of pRb has been mapped to the C-terminal region downstream from the pockets. In this complex, pRb appeared to regulate the kinase activity of c-Abl during the cell cycle.

We have reported previously that one of the 70-kDa heat shock protein families, hsc73, could interact with pRb in vivo (16). Our present data showed that hsc73 could interact directly with pRb in vitro and that the interaction was specific since neither hsp90 nor m65hsp could associate with pRb. The mapping of the hsc73 binding site of pRb revealed a novel region within the N-terminal region upstream from the pockets. The binding of hsc73 to pRb was unaffected by the viral oncoprotein E1A, suggesting that there might be a different biological role for this interaction.

Protein interactions depend on the unique amino acid motif to interact with each other specifically. BIP, the sole member of the hsp70 family localized in the endoplasmic reticulum of eukaryotic cells, is known to recognize polypeptides that contain a heptameric motif best described as HyXHyXHyXH, where Hx is a large hydrophobic amino acid and X is any amino acid (27). The N-terminal 301–372 amino acid residues outside the A/B pocket of pRb contain this heptameric motif (residues 331–337). Since the substrate binding domains of BIP and hsc73 are suggested to have the identical structure, it can be speculated that hsc73 binds to pRb by recognizing this motif.

We need to consider one other possible explanation for deletion mutant binding studies. Members of the hsp70 family appear capable of recognizing “nonnative” form of proteins (28). Consequently one could argue that the differential binding observed in this study simply represents mutants that have folded either into a native or nonnative-like configuration. Indeed this is a problem that will always have to be considered regarding proteins that bind stably to members of the molecular chaperone family. However, in this present study we showed that all deletion mutants that contain pRb301–372 could bind to hsc73, whereas no deletion mutants lacking this region could bind to hsc73. It is highly unlikely that all mutant proteins that contain pRb301–372 become nonnative or malformed proteins and consequently bind to hsc73 and vice versa. Therefore, our present studies strongly indicate that the primary pRb 301–372 sequence is important for binding to hsc73.

Meanwhile, we also have to mention the stoichiometry or affinity of the pRb and hsc73 interaction. One of the key observations is presented in Fig. 4B on the interaction between hsc73 and a specific region of pRb. In these experiments, 1 µg of the various GST-del Rb constructs is incubated with 1–2 µg of hsc73 and the complexes detected by immunoprecipitation and Western blot analysis. Lane 1 of Fig. 4B corresponds to the input amount of hsc73, therefore comparison with the other lanes gives some indication of the stoichiometry or affinity of pRb and hsc73 interaction. It is indicated that some of the bands are very weak (lane 6). This could be interpreted that the interaction is either of low affinity, that the GST-del Rb proteins are heterogeneous resulting in reduced stoichiometry, or that it is a malformed subpopulation of the GST-del Rb proteins which interacts with hsc73. Further experiments are required to clarify each of these possibilities.

It is known that hsp70 family act as molecular chaperones (29, 30). Hsp70 families can associate physically with various intracellular proteins and work for the regulation of conformational changes, translocation, and stabilization of these proteins. Although the functional significance of hsp70 families for cell growth or malignant transformation has not been clarified yet, it has been reported that overexpression of hsc73 could
suppress oncogene-mediated transformation (31). Therefore it is speculated that hsc73 can function as a tumor suppressor in the process of transformation and that this function may be mediated by the pRb through their physical association. Hsc73 may change the conformation of pRb so that pRb becomes resistant to phosphorylation since phosphorylation of pRb results in the dissociation of the pRb-E2F complex and the loss of the growth-suppressive effect. We tested the susceptibility of pRb to phosphorylation in vitro by p34cdc2 kinase in the presence of hsc73. However, there was no change in the pRb phosphorylation (data not shown). It is noteworthy that another tumor suppressor, p53, is also associated with hsc73 (32–35) and that the interaction is mediated by the N-terminal region of p53 (36). Therefore, it is speculated alternatively that hsc73 may stabilize the nonphosphorylated or the hypophosphorylated pRb and extend its half-life in a manner analogous to that of mutant p53.

None of the pRb-binding proteins has been shown to bind to hyperphosphorylated pRb so far. Unexceptionally, hsc73 could not interact with the hyperphosphorylated form of pRb, suggesting a regulatory mechanism of the hsc73-pRb interaction similar to that of other pRb-binding proteins. It has been shown that pRb can be phosphorylated at several serine or threonine residues in various regions. The hsc73 binding region also includes at least two threonine residues, which could become a substrate for cyclin-dependent kinase (37–40), indicating that the dissociation might depend on the direct phosphorylation of the binding region rather than on the conformational change following phosphorylation of other regions. Interestingly, the hsc73-pRb complex could be disrupted by the addition of a high concentration of ATP. ADP could not induce the disruption efficiently, suggesting that the dissociation might be mediated by ATP hydrolysis by hsc73. However, as shown in Fig. 2, lane 5, we noted that the addition of the nonhydrolyzable ATP analog ATPγS did in fact result in considerable dissociation of the complex. This may be consistent with work by Palleros et al. (41), suggesting that it is ATP exchange rather than ATP hydrolysis.

Finally, two important questions remain to be answered. What is the biological significance of the formation of the hsc73-pRb complex? What is the functional significance of the ATP-dependent disruption of the complex in the cell cycle? Gene transfer experiments using mutant hsc73 or mutant pRb may help to resolve some of these questions.

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