Disruption of the interaction between retinoblastoma protein and 70 kD heat shock protein leads to growth acceleration of tumor cells

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ABSTRACT

Retinoblastoma protein (pRb) is a phosphoprotein regulating cell growth. During G1 phase in the cell cycle, pRb is dephosphorylated and inhibits the progression to S phase. Previously we demonstrated that 70 kDa heat shock protein (HSP70) was associated with dephosphorylated pRb *in vivo* and *in vitro,* and mapped the HSP70 binding region of pRb. In this report we analyzed the consequences of disruption of the HSP70-pRb interaction in cells. Recombinant fusion proteins containing the 11 amino-acid HIV TAT transduction domain and HSP70 binding domain (residues 301-372, RbHBD) or N-terminal non-binding region of pRb (RbNTR) were produced. The TAT fusion proteins were introduced into cells by addition into culture medium. TAT-RbHBD protein, but not TAT-RbNTR protein, was capable of binding to HSP 70 in the cells. Transduction of TAT-RbHBD protein inhibited the association of HSP70 to pRb in cells; however, TAT-RbNTR fusion protein failed to do so. Strikingly, transduction of TAT-RbHBD fusion protein promoted proliferation of HOS cells, Jurkat cells and U-937 cells expressing functional pRb, but not of SAOS cells lacking pRb. In addition, HOS cells treated with heat shock became more resistant to the effect of TAT-RbHBD protein. These data indicate that disruption of the HSP70-pRb interaction leads to growth acceleration of cells. Our study revealed an important *in vivo* role of HSP70-pRb interaction in cell cycle control.

Key words : Retinoblastoma protein, HSP70, TAT, Heat shock, Cell proliferation

INTRODUCTION

HSP70 is a molecular chaperone which assists folding, assembly and sorting of various cellular proteins in the cytosol and nucleus. In response to stress such as heat shock, the expression of HSP70 increases and it translocates from cytosol to nucleus. It is known that a variety of stress can induce growth suppression in mammalian cells. However, little is known about the molecular mechanism of stress-

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induced growth suppression. Some of the HSPs are apparently involved in the regulation of the cell cycle, since there have been a number of reports demonstrating the direct binding of HSPs to critical cell cycle regulators [1–5].

Previously we showed that HSP70 could bind to pRb *in vivo* and *in vitro* [6]. pRb was first identified as a tumor suppressor protein, and loss of functional pRb by deletions, mutations or DNA viral protein leads to oncogenic transformation [7]. pRb is also important for cell survival, since loss of pRb leads to apoptosis in certain cells [8–11]. The function of pRb is regulated by phosphorylation. Dephosphorylated pRb, a functional form of pRb, binds to transcriptional factor E2F, leading to transcriptional suppression and G1 arrest in the cell cycle, whereas phosphorylation of pRb by cyclin-dependent kinases leads to dissociation from E2F, allowing it to initiate transcription of the target genes and cell cycle progression [12]. Importantly, it was revealed that HSP70 could selectively bind to dephosphorylated pRB, but not to phosphorylated pRb [4]. The HSP70-binding region of pRb was mapped in the amino-terminal region (residues 301–372) adjacent to the so called A pocket region [4]. As a result of examination of the functional significance of the HSP 70-pRb interaction *in vitro*, it was demonstrated that HSP70 and cochaperone HSP40 prevented aggregation of purified dephosphorylated pRb and protected it from degradation in vitro. Gene transfer-mediated overexpression of HSP70 in HOS cells decreased the growth rate of transfected cells. These findings suggested that HSP70 might assist the growth inhibitory role of dephosphorylated pRb and thus encouraged us to examine whether disruption of the HSP70-pRb interaction could affect cell growth.

In this study we used a technology to introduce recombinant proteins into cultured cells. HIV TAT protein is capable of penetrating cell membranes [13, 14], and the membrane transduction domain consisting of 11 amino acids was identified in the protein [15]. Though the exact mechanism of transduction across cell membranes is still unknown, a number of reports have shown the efficient introduction of recombinant TAT fusion proteins or peptides into cultured cells and animal tissues [15–18]. We constructed bacterial expression vectors to produce recombinant TAT fusion proteins containing either HSP70 binding domain (RbHBD) or N-terminal non-binding region (RbNTR) of pRb. The TAT fusion proteins were readily transduced into cells, and TAT-RbHBD fusion protein, but not TAT-RbNTR fusion protein bound to HSP70 in cells, inhibited the HSP70-pRb association and enhanced proliferation of human tumor cell lines expressing functional pRb. A novel and important role of HSP70 in cell growth control is shown and discussed in this paper.

MATERIALS AND METHODS

Construction of bacterial expression vector coding TAT fusion proteins

A bacterial expression vector pET-TAT was constructed by insertion of a double-stranded oligonucleotide encoding the TAT transduction domain (YGRKKRRQRRR) into the BamHI and EcoRI site of pET21a vector (Novagen, Madison, WI), followed by insertion of NdeI -XhoI fragment of the resulting pET21a-TAT vector into pET15b vector (Novagen).
The vector pET-TAT has an N-terminal 6 histidine tag followed by a T7 tag, TAT transduction domain and a multicloning site (Fig. 1).

DNA coding HSP70 binding domain of pRb (residues 301–372, RbHBD) and N-terminal region (residues 1–300, RbNTR) were generated by PCR. Briefly, a plasmid p4.95BT (from Dr. T. P. Dryja, Harvard Medical School) containing full-length pRb coding region was subjected to amplification using two primer sets (a forward primer 5'-TCGAGCTCTGAATTCTCTTGG-3' and a reverse primer 5'-CACTCGAGTGTGTGGAGG-3' to generate RbHBD-coding DNA; a forward primer 5'-TCGAGCTCTCATGCCGCCC-3' and a reverse primer 5'-CACTCGAGAATTCATAA-3' to generate RbNTR-coding DNA) designed to

include *SacI* site and *XhoI* site at the 5'-end and 3'-end, respectively. PCR was carried out using KOD plus DNA polymerase (TOYOBO, Osaka, Japan) for 35 cycles (1 min at 96℃, 0.5 min at 60℃, and 1 min at 68℃ for amplification of RbHBD-coding DNA, 0.5 min at 94℃, 0.5 min at 55℃, and 0.5 min at 72℃ for amplification of RbNTR-coding DNA, followed by a final 7 min incubation at 72℃) using a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) The resulting 216 bp and 900 bp PCR products were gel-purified, digested by SacI and XhoI and then subcloned in-flame into the *SacI* and *XhoI* sites of the plasmid pET-TAT. Sequencing of the insert in these plasmid constructs confirmed the exact sequence coding TAT-RbHBD or TAT-RbNTR fusion protein with no artifactual PCR-mediated mutations elsewhere.

Expression and purification of TAT fusion proteins

Expression of TAT fusion proteins was performed in the same manner as described previously [4]. Briefly, 500 ml cultures of *Escherichia Coli* (strain BL21 [DE3], Novagen) transformed with pET-TAT-RbHBD or pET-TAT-RbNTR vector were supplemented with isopropyl-1-thio-b-D-galactopyranoside (Wako fine chemicals, Japan) to a final concentration of 1 mM, followed by incubation for 2 h at 37℃ with shaking. Bacteria were then pelleted and frozen at -80℃.

Purification of the recombinant fusion proteins was performed as described by Nagahara et al. [15]. Bacterial pellets were thawed, resuspended in 10 ml of buffer A (8 M urea, 20 mM HEPES [pH 8.0], 100 mM NaCl) and lysed on ice by sonication, followed by centrifugation at 16000 x g for 30 min at 4℃. Cellular lysates were loaded onto a 5 ml Ni-NTA column (Qiagen, Valencia, CA, USA) which was pre-equilibrated with buffer A containing 10 mM imidazole. The column was washed with buffer A and fusion proteins were eluted with buffer A containing 100 mM imidazole. 1 ml fractions were collected and analyzed for protein concentrations by a spectrophotometer (Beckman DU530, USA). 10 μl of each fraction was analyzed by 10 % SDS polyacrylamide gel electrophoresis (PAGE), followed by staining with Coomassie Brilliant Blue. Fractions con-

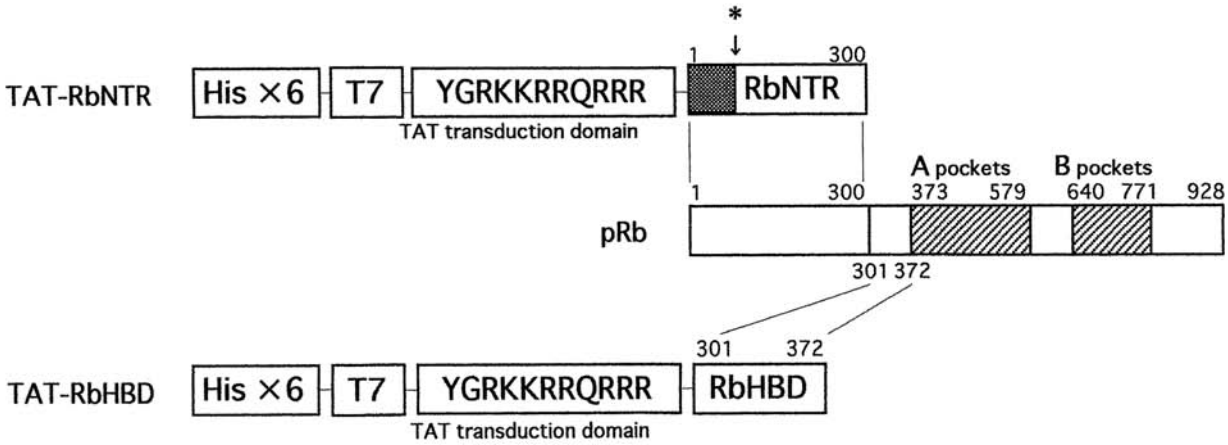

Fig.1 Structure of recombinant TAT fusion proteins

His x 6 and T7 indicate 6-histidine tag and T7 tag, respectively, and are derived from pET15b and pET21a vectors (Novagen), respectively. RbHBD and RbNTR indicate HSP70 binding domain (amino acids 301-372) and N-terminal non-binding region (amino acids 1-300) of human pRb, respectively. An asterisk indicates the possible cleavage site within RbNTR region (amino acids 50-60) estimated from the molecular weight of purified proteins.

taining fusion proteins were dialyzed against PBS for 18 h at 4℃. Aliquots of purified fusion proteins were analyzed by 8 % SDS-PAGE, followed by staining with Coomassie Brilliant Blue or immunoblotting.

Cell culture

Human HOS osteosarcoma cells, pRb-deficient SAOS osteosarcoma cells, Jurkat T-lymphoma cells and U-937 histiocytic leukemia cells were obtained from American Tissue Type Collection. HOS cells were cultured in RPMI1640 (GIBCO BRL, Grand Island, NY, USA) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 10 % fetal calf serum (FCS, Filtron, Brooklyn, Australia). All other cells were grown in Dulbecco's modified minimum essential medium (DMEM, GIBCO BRL) supplemented with 10 % FCS, 2 mM L-glutamine and antibiotics.

Antibodies and purified proteins

Anti-HSP70 monoclonal antibody 1B5 was kindly provided by Dr. Kenzo Ohtsuka of Aichi Cancer Research Center. Anti-HSP70 monoclonal antibody 3a3 was purchased from Affinity BioReagents (Neschanic Station, NJ). Anti-pRb monoclonal antibodies G3-245 (reacting to amino acids 324-344 of human pRb), G99-2005 (reacting to amino acids 1-240 of human pRb) and G 99-549 (reacting to amino acid 514-610 of human pRb) were purchased from Pharmingen (San Diego, CA, USA). Anti-T7 monoclonal antibody was purchased from Novagen.

Bovine brain HSP70 were purchased from StressGen (Victoria, BC, Canada) and Pharmingen, respectively.

Confocal laser microscopy and flow cytometry

For confocal laser microscopy, cells were grown on collagen-coated cover slips (Biocoat; Becton Dickinson, San Jose, CA, USA). HOS cells or Jurkat cells were incubated with culture medium supplemented with or without 500 nM of TAT fusion proteins for 2 h at 37℃, washed in PBS, fixed in fixation buffer (1 % paraformaldehyde/10 mM EDTA/PBS) for 15 min at 4℃, and permiabilized with 0.1 % Triton-X100/10 mM EDTA/PBS for 10 min. After washing once in washing buffer (0.01 % Triton X100/10 mM EDTA/PBS), cells were incubated with anti-T7 antibody for 40 min at 4℃, washed in washing buffer and incubated with FITC-labeled goat anti-mouse IgG+IgM antibody (Kirkegaard & Perry Lab.[KPL], Gaithersburg, MD, USA) for 40 min, followed by analysis using an MRC1024ES confocal laser microscope (Bio-Rad, Hercules, CA, USA) or FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Immunoprecipitation and Immunoblotting

2 x 10^7 HOS cells grown on a dish were incubated with or without 1 μg/ml of aphidicolin (Sigma, St. Louis, MO, USA) and 500 nM TAT fusion protein for 24 h at 37℃. For immunoprecipitation, cells were harvested, washed twice in ice-cold PBS, and lysed in 1.0 ml lysis buffer (0.5 % CHAPS /50 mM Tris-HCl [pH 7.5] /150 mM NaCl) including protease inhibitor cocktail (Boehringer Mannheim, Germany). After incubation on ice for 45 min, nuclei and cell debris were removed by centrifugation (12000 x g, 10 min). The lysates were incubated with 2 μg of anti-pRb antibody and 50 μl anti-mouse IgG agarose beads (Sigma) for 18 h at 4℃. After washing four times with lysis buffer, immunoprecipitates were boiled for 5 min in reducing SDS sample buffer (2 % SDS, 1 % 2-mercaptoethanol, 5 % glycerol, 125 mM Tris-HCl [pH 6.8] and 0.003 % bromophenol blue).

For detection of pRb in HOS cells, cell lysates were obtained by lysis in RIPA buffer (1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 158 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl [pH 7.2]) supplemented with protease inhibitors (0.1 TIU aprotinin, 1 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, 10 μg/ml leupeptin), incubated for 10 min at 4℃, and centrifuged (14000 x g, 30 min). Protein concentrations in the lysates were analyzed by

micro BCA assay (Pierce, Rockford, IL, USA).

Cell lysates, immunoprecipitates or purified proteins were boiled for 5 min in reducing SDS sample buffer, separated by 8 % SDS-PAGE and transferred onto Immobilon membranes (Millipore, Bed ford, MA, USA). The membranes were soaked in blocking buffer (10 % non-fat dry milk, PBS) for 1 hour at room temperature. Then, the membranes were incubated for 90 min with anti-pRb antibody or anti-HSP70 antibody 3a3. After washing in PBS containing 0.1 % Tween-20, the membranes were incubated with horseradish peroxidase-labeled goat anti-mouse IgG antibodies (KPL) for 30 min, washed, and soaked in ECL detection fluid (Amersham, Birmingham, AL, USA) for one min. The bands were visualized using X-ray films (Fuji Photo Film, Tokyo, Japan).

in vivo binding assay of TAT fusion proteins to HSP70

2 x 10^7 HOS cells were cultured in the complete medium supplemented with 500 nM TAT fusion proteins for 24 h at 37℃. Cells were harvested, washed twice in ice-cold PBS, and lysed in 1.0 ml CHAPS lysis buffer. After incubation on ice for 10 min, nuclei and cell debris were removed by centrifugation (12000 x g, 10 min).

The lysates were incubated with 50 μl Ni-NTA agarose beads (Qiagen) which had been washed in CHAPS lysis buffer containing 10 mM imidazole. After vigorous rotation for 3 h at 4℃, the beads were washed four times with CHAPS lysis buffer and boiled for 5 min in reducing SDS sample buffer. Proteins precipitating with the beads were separated by 8% SDS-PAGE and subjected to immunoblotting with anti-T7 antibody or anti-HSP70 antibody.

[^{3}H]-thymidine uptake assay

HOS cells or SAOS cells (4 x 10^4/well) were plated in 96-well dishes and allowed to adhere overnight in the complete medium. The medium was then discarded, and the cells were incubated at 37 ℃ in a medium supplemented with or without TAT fusion proteins at a final concentration of 500 nM . In some cases, cells were incubated for 2 h at 42℃ on a water bath at 2 h after the supplementation. Cells were then pulsed with 1 μCi/well [^{3}H]-thymidine, incubated for additional 12 h at 37 ℃ and frozen. Following thaw and harvest of cells, radioactivities of [^{3}H]-thymidine taken up by cells were analyzed by a BECKMAN LS6000LL liquid scintillation counter (Beckman, Fuleerton, CA, USA).

RESULTS

Generation of TAT-RbHBD and TAT-RbNTR fusion proteins

In our previous study, it was shown that HSP70 binds selectively to dephosphorylated pRb in the amino-terminal region (amino acids 301-372) outside the so called A pocket region [4]. In order to disrupt the HSP70-pRb interaction, we attempted to introduce a deletion mutant pRb protein consisting of the HSP70-binding domain (RbHBD) into cells. RbHBD protein was expected to inhibit the binding of HSP70 to pRb competitively. Another deletion mutant pRb protein which consists of N-terminal non-HSP 70 binding region (RbNTR; amino acids 1-300) [4] was also designed as a control study (Fig. 1).

A new technology was developed for transduction of recombinant fusion proteins into cytosol [15]. HIV TAT protein-derived sequence YGRKKRRQRRR could direct an efficient transduction of various proteins across cell membranes. Based on these reports, we constructed bacterial expression vectors to produce recombinant TAT fusion proteins containing either RbHBD or RbNTR. The vectors, pET-TAT-RbHBD and pET-TAT-RbNTR have an N-terminal 6-histidine tag followed by T7 tag, TAT transduction domain and RbHBD and RbNTR respectively (Fig. 1). Fusion proteins were produced by bacteria, purified by Ni-NTA agarose beads in a urea-denaturing condition and characterized by 12 % SDS-PAGE, followed by staining with Coomassie Brilliant Blue (Fig. 2 A) and immunoblotting with anti-pRb antibodies (Fig. 2B and 2C).

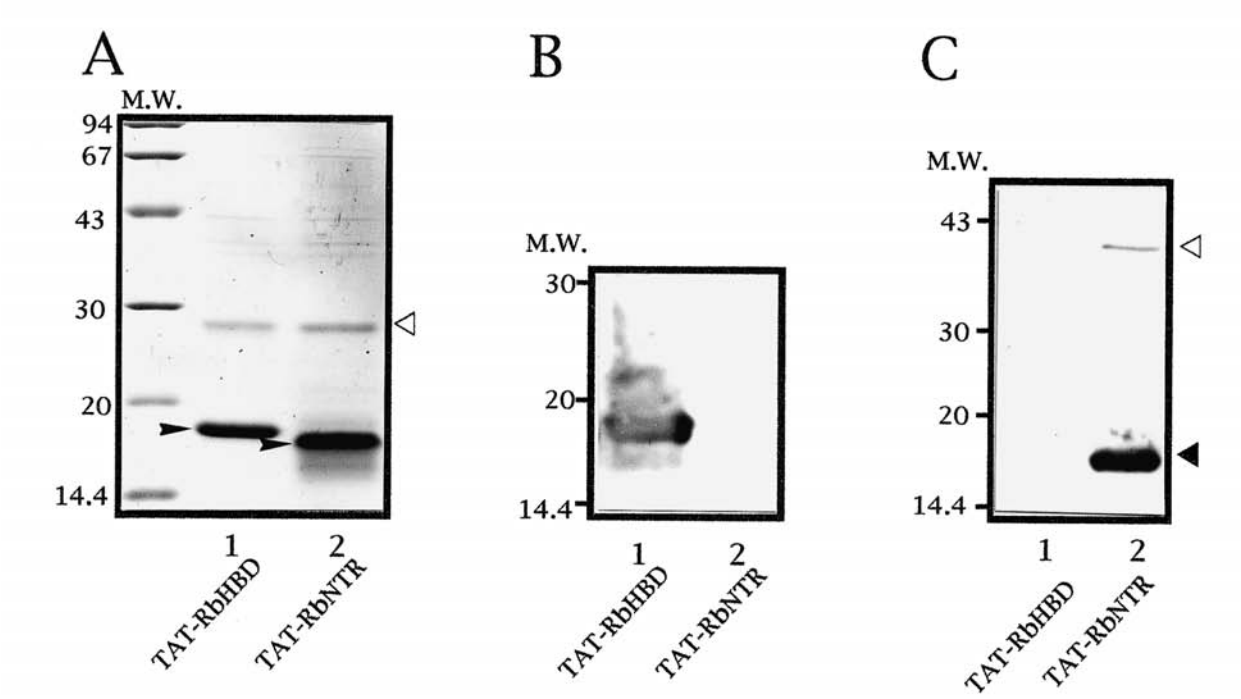

Fig.2 Biochemical characterization of TAT fusion proteinss

Panel A: Proteins purified by Ni-NTA agarose column were separated by 12 % SDS-PAGE, followed by Coomassie Brilliant Blue staining. 18 kD TAT-RbHBD protein (lane 1) and 17 kD TAT-RbNTR protein (lane 2) protein are indicated by closed arrow heads. Ni-NTA-binding non-specific protein (lane 1 and 2) is indicated by an open arrow head. Panel B: TAT fusion proteins were separated by 12 % SDS-PAGE, followed by immunoblotting with anti-pRb monoclonal antibody G3-245 (reacting amino acids 324-344 of human pRb). Panel C: TAT fusion proteins were separated by 12 % SDS-PAGE, followed by immunoblotting with anti-pRb monoclonal antibody G99-2005 (reacting to amino-acids 1-240 of human pRb). 17 kD cleaved form and smaller amount of 41 kD non-cleaved form of TAT-RbNTR fusion proteins are indicated by closed arrow head and open arrow head, respectively.

Expected 18 kD TAT-RbHBD fusion protein was demonstrated by protein staining (Fig. 2A, lane 1). TAT-RbNTR protein, however, was cleaved within N-terminal region of RbNTR (estimated amino acids 50-60) by some bacterial proteases, resulting in 17 kD fusion protein (Fig. 2A, lane 2), shorter than the expected size (approximately 41 kD). A smaller amount of 28 kD protein included in both purified proteins was likely to be Ni-NTA-binding non-specific protein derived from bacteria. The TAT-RbHBD fusion protein was detected by anti-pRb monoclonal antibody G3-245 reacting to amino-acids 324-344 of human pRb, while the TAT-RbNTR fusion protein was not (Fig. 2B). In contrast, anti-pRb monoclonal antibody G99-2005 reacting to amino-acids 1-240 of human pRb was capable of detecting the 17 kD cleaved form and a smaller amount of the 41 kD non-cleaved form of TAT-RbNTR fusion protein, but failed to detect TAT-RbHBD fusion protein (Fig. 2C). This also indicates that the epitope defined by G99-2005 is located within N-terminal 1-60 amino acids of human pRb. Both recombinant fusion proteins remained soluble in PBS after removal of urea by rapid dialysis.

Transduction of TAT fusion proteins into cells

The ability of TAT fusion proteins to transduce into cells was analyzed by confocal laser microscopy and flow cytometry. HOS cells were incubated in a complete medium containing 500 nM of TAT-RbHBD or TAT-RbNTR fusion protein, fixed, permiabilized and stained with anti-T7 antibody and FITC-labeled anti-mouse Ig antibody. Confocal laser microscopy analysis showed that TAT-RbHBD and TAT-RbNTR fusion proteins were readily transduced into cells after incubation for 2 h at 37℃ and were localized diffusely in cytosol and nucleus (Fig. 3). Intracellular FACS analysis after incubating Jurkat cells with or without 500 nM TAT-RbHBD or TAT-RbNTR fusion protein demonstrated that similar amounts of proteins were transduced into these cells (Fig. 4) and U-937 cells (data not shown). Since TAT fusion protein was not detected on cell surface by FACS analysis of non-permiabilized cells (data not shown), increased fluorescence intensity as shown in Figure 4 does not represent only non-specific attachment to outer cell membrane.

TAT-RbHBD protein could bind to HSP70 in HOS cells

In order to show that TAT-RbHBD, but not TAT-RbNTR, is capable of binding to HSP 70, HOS cells incubated with 500 nM TAT-RbHBD or TAT-RbNTR fusion protein for 2 h were lysed and incubated with Ni-NTA agarose beads. Proteins precipitated with the beads were eluted by 250 mM imidazole, separated by SDS-PAGE and detected by immunoblotting. TAT-RbHBD and TAT-RbNTR fusion proteins were detected by anti-T7 antibody at the 18 kD band and 17 kD band, respectively (Fig. 5 A). Immunoblotting using anti-HSP70 antibody 3a3 demonstrated co-precipitation of HSP70 with TAT-RbHBD, whereas no band was detected in cells incubated with TAT-RbNTR or without TAT fusion protein (Fig. 5B). The amount of HSP70 precipitated with TAT-RbHBD was relatively small as compared to the level of HSP70 in cell lysate. It is reasonable because there may be a large number of HSP70 associated with other proteins in higher affinity than with TAT-RbHBD protein and pRb. These data confirmed that TAT-RbHBD fusion protein transduced into cells was capable of

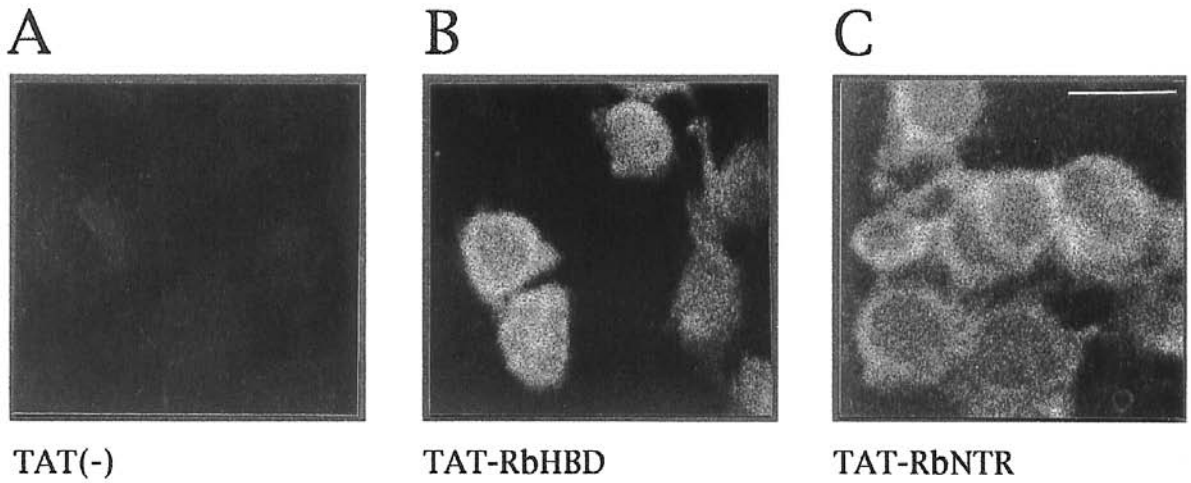

Fig.3 Confocal laser microscopic analysis of transduced proteins

HOS cells were incubated with a culture medium alone (panel A), 500 nM TAT-RbHBD protein (panel B) or 500 nM TAT-RbNTR protein (panel C) for 2 h. Cells were washed, fixed, permiabilized and incubated with anti-T7 antibody and FITC-labeled goat anti-mouse IgG+ IgM antibody, followed by confocal laser microphotography (x 800 magnification). Bar, 25 μm.

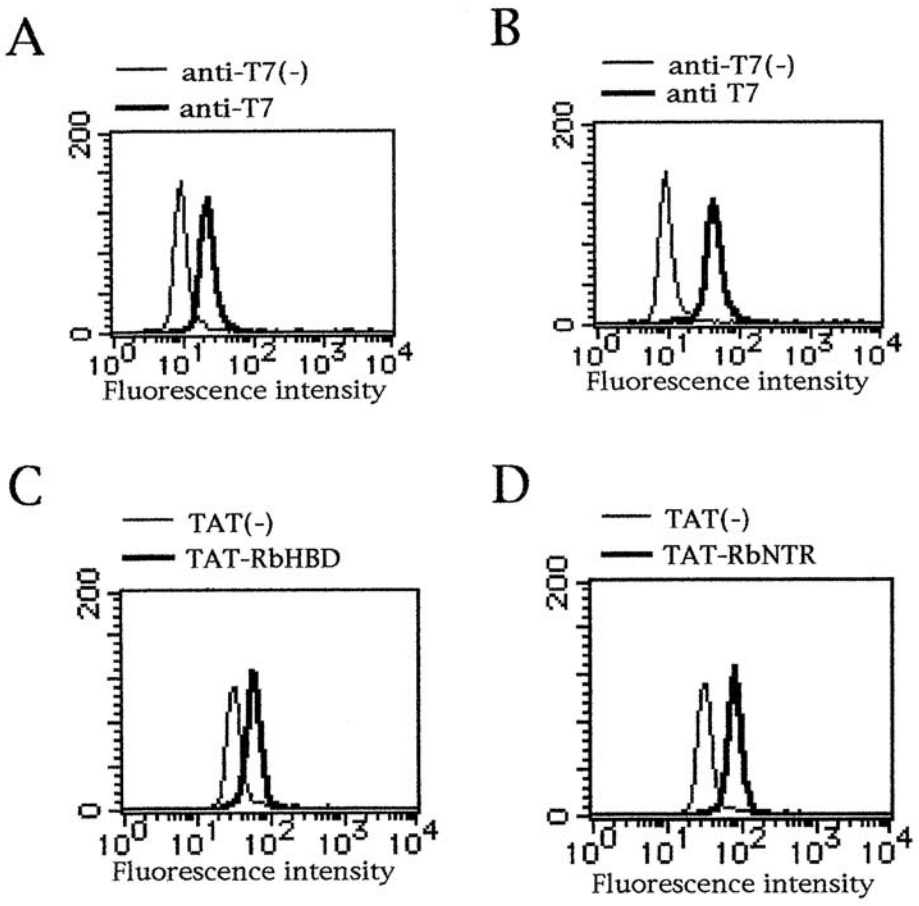

Fig.4 FACS analysis of intracellular TAT fusion proteins

Jurkat cells were incubated with 500 nM TAT-RbHBD protein (panels A and C) or 500 nM TAT-RbNTR protein (panels B and D) for 2 h. Cells were washed, fixed, permiabilized and incubated with anti-T7 antibody and FITC-labeled goat anti-mouse IgG+IgM antibody, followed by flow cytometry. In panel A and B, thick line and thin line indicate cells incubated with or without anti-T7 antibody, respectively. In panels C and D, thick line and thin line indicate cells incubated with or without TAT fusion proteins, respectively.

binding to HSP70, and this was consistent with our previous *in vitro* study [4].

TAT-RbHBD protein inhibited the association of HSP70 with pRb in HOS cells

It was shown previously that RbHBD was the only domain of pRb associated with HSP70[4]. Therefore it was expected that transduction of TAT-RbHBD protein would be able to competitively inhibit the HSP70-pRb interaction in cells. Since the level of dephosphorylated pRb, to which HSP70 could bind, was very low in HOS cells in our preliminary experiments, cells were treated with aphidicolin for 24 h in order to increase the level of dephosphorylated pRb. Then, HOS cells were incubated with TAT-RbHBD or TAT-RbNTR protein for 12 h, lysed in CHAPS lysis buffer and subjected to immunoprecipitations with anti-HSP70 antibody 1B5 or anti-pRb antibody G99-549 reacting only to endogenous 110 kD pRb. The immunoprecipitated proteins were separated by SDS-PAGE and detected by immunoblotting with anti-HSP70 antibody 3a3 or anti-pRb antibody G3-245.

Similar levels of HSP70 were precipitated

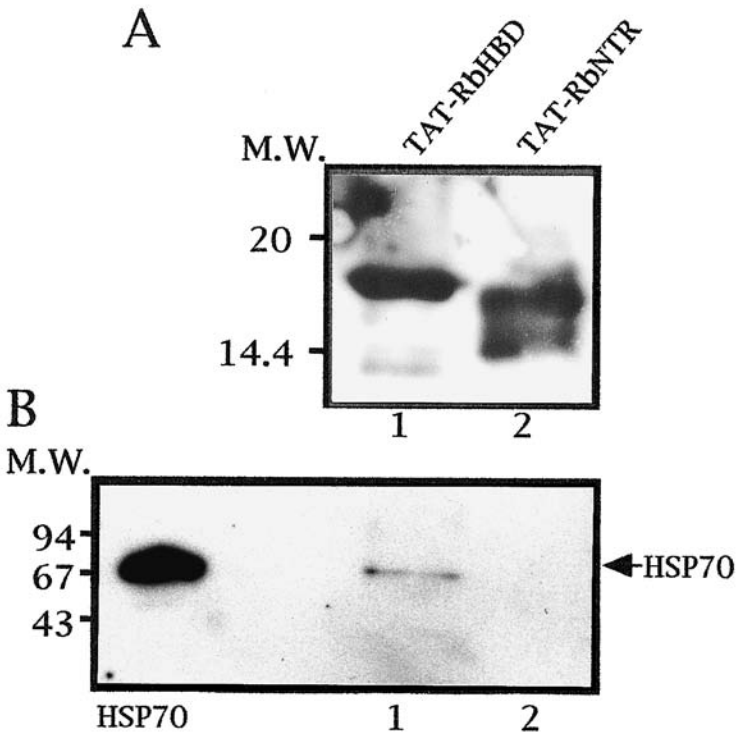

Fig.5 TAT-RbHBD protein binds to HSP70 in HOS cells

HOS cells incubated with 500 nM TAT fusion proteins were lysed and incubated with Ni-NTA agarose beads.

Panel A: Proteins precipitated with the beads from TAT-RbHBD-transduced cell lysate (lane 1) or TAT-RbNTR-transduced cell lysate (lane 2) were eluted by 250 mM imidazole, separated by 12 % SDS-PAGE and detected by immunoblotting with anti-T7 antibody.

Panel B: 1 mg purified HSP70 (left lane) or proteins precipitated with the beads from TAT-RbHBD-transduced cell lysate (lane 1) or TAT-RbNTR-transduced cell lysate (lane 2) were eluted by 250 mM imidazole, separated by 8 % SDS-PAGE and detected by immunoblotting with anti-HSP70 antibody 3a3. HSP70 is indicated by an arrow.

from HOS cells transduced with TAT-RbHBD and from those transduced with TAT-RbNTR fusion proteins (Fig. 6A). The data indicated that TAT fusion protein could not affect the relative expression level of HSP70 in HOS cells transduced with either TAT fusion protein. Similar levels of endogenous 110 kD pRb and phosphorylated pRb (ppRb) were detected in both cell lysates (Fig. 6B). However, far smaller amounts of HSP70 was detected in the pRb-immunoprecipitates from cells transduced with TAT-RbHBD fusion protein (Fig. 6C). Densitometric analysis demonstrated that the level of HSP70 precipitated with pRb from TAT-RbHBD-transduced cells was approximately one eighth of that from TAT-RbNTR-transduced cells. These results indicate that transduced TAT-RbHBD protein could block the association of HSP70 with pRb in cells.

Transduction of TAT-RbHBD protein promoted proliferation of cells expressing pRb

In order to know the biological effect of disruption of the HSP70-pRb association on the cell, we focused on cell proliferation, since pRb is one of the major tumor suppressor proteins.

[^{3}H]-thymidine uptake assay was performed using HOS cells incubated with or without TAT fusion proteins. Strikingly, HOS cells transduced with TAT-RbHBD fusion protein displayed increased [^{3}H]-thymidine uptake after 12 h incubation, whereas TAT-RbNTR fusion protein did not affect the proliferation of HOS cells (Fig. 7A, lanes 4-6). Since heat shock can increase the amount of HSP70 protein, we examined the effect of heat stress treatment on the TAT-RbHBD-mediated promotion of cell proliferation. When HOS cells were incubated with TAT fusion proteins at 42℃ for 2 h, they became less sensitive to TAT-RbHBD fusion pro-

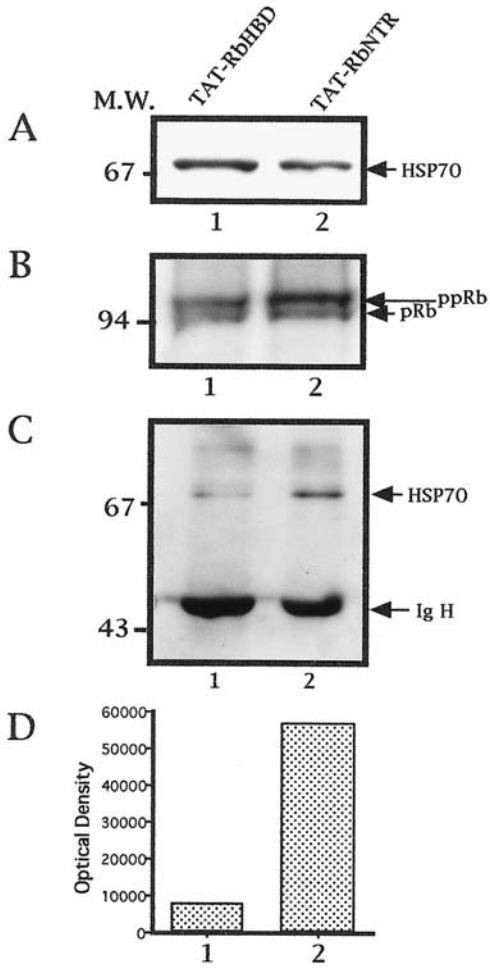

Fig.6 TAT-RbHBD protein inhibits the HSP70-pRb association in HOS cells

HOS cells were treated with aphidicolin for 24 h, incubated with TAT fusion proteins for 2 h, lysed and subjected to immunoprecipitations. Proteins precipitated from TAT-RbHBD-transduced cell lysate (lane 1) or TAT-RbNTR-transduced cell lysate (lane 2) were separated by 8 % SDS-PAGE and detected by immunoblotting. Panel A: Proteins precipitated with anti-HSP70 antibody 1B5 were detected by immunoblotting with anti-HSP70 antibody 3a3. HSP 70 is indicated by an arrow. Panel B: Proteins in cell lysates were detected by immunoblotting with anti-pRb antibody G3-245. Phosphorylated pRb (ppRb) and dephosphorylated pRb (pRb) are indicated by arrows. Panel C: Proteins precipitated with anti-pRb antibody G99-549 were detected by immunoblotting with anti-HSP70 antibody 3a3. HSP70 and Ig heavy chain (IgH) are indicated by arrows. Panel D: The density of HSP70 bands in panel C was quantitated by densitometric analysis. Vertical axis indicates the densitometric value of the HSP70 band calculated by NIH image freeware program.

tein than non-stress cells (Fig. 7A, lanes 1-3). Then, we examined the effect of transduction of TAT fusion proteins into SAOS osteosarcoma cells which lacked functional pRb. In contrast to the effect on HOS cells, TAT-RbHBD fusion protein could not affect proliferation of SAOS cells with or without heat stress treatment (Fig. 7B). Thus, disruption of the HSP70-pRb association appeared to lead to accelerated proliferation of cells expressing functional pRb.

We assessed further whether above findings in HOS cells would be generalized to other type of cells. Jurkat T-lymphoma cells and U-937 histiocytic cells also express functional pRb (data not shown). Since both TAT-RbHBD and TAT-RbNTR fusion proteins are transduced into these cells as well as into HOS cells (Fig. 4), we examined whether transduction of the TAT fusion proteins could modulate growth rates of these cells. Cells were cultured for 3 days in the absence or presence of 500 nM TAT-RbHBD or TAT-RbNTR fusion proteins in culture media and cell numbers were counted. It was clearly demonstrated that transduction of TAT-RbHBD, but not of TAT-RbNTR, accelerated the growth rate of both Jurkat cells and U-937 cells.(Fig. 8).

DISCUSSION

pRb is a tumor suppressor gene product regulating cell cycle progression, especially at the G1-S entry [7]. The function of pRb is regulated mostly by phosphorylation. Dephosphorylated pRb suppresses the transcriptional activity of E2F by binding directly through the so-called pocket regions, thus acting as a major inhibitor of progression from G1 to S in the cell cycle. We have previously shown that HSP70, one of

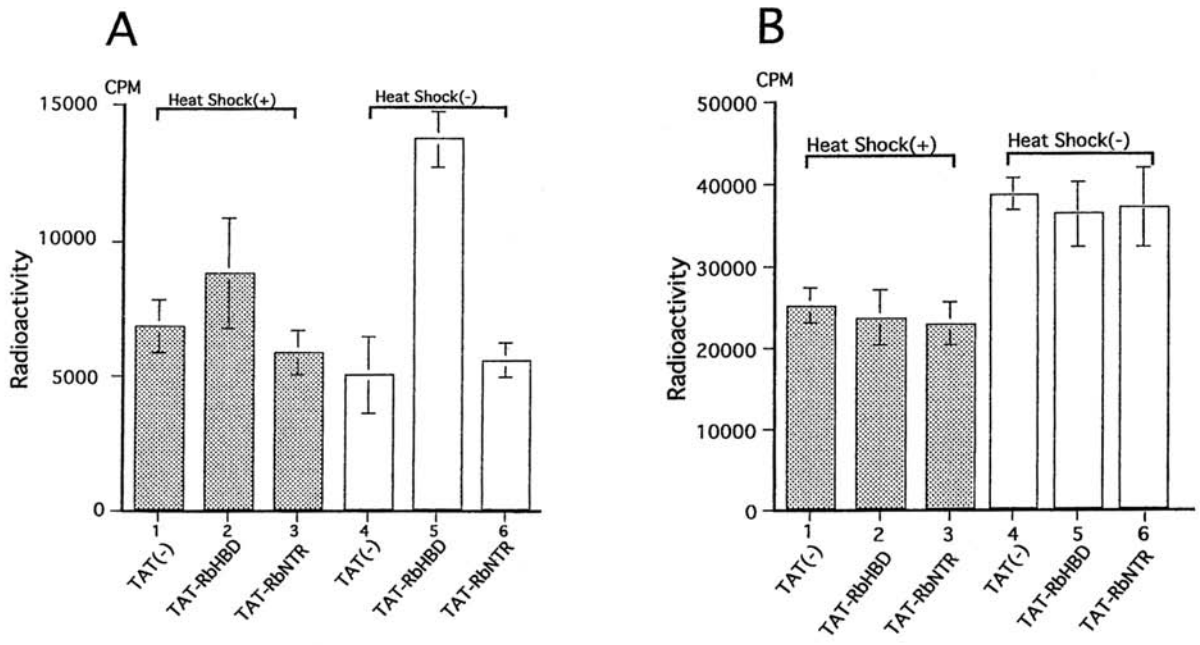

Fig.7 Effect of transduction of TAT fusion proteins on proliferation of HOS and SAOS cells

HOS cells (panel A) or SAOS cells (panel B) were cultured in the absence (lane 1 and 4) or presence of 500 nM TAT-RbHBD (lane 2 and 5) or TAT-RbNTR (lane 3 and 6) protein at 37 ℃. In some cases, cells were incubated at 42℃ for 2 h after the addition of TAT fusion proteins into culture medium (lanes 1-3). Cells were then pulsed with [3H]-thymidine and incubated for an additional 12 h at 37℃, followed by scintillation counting of cells. Vertical axes represent the radioactivities (count per minute).

the molecular chaperones, was associated directly with dephosphorylated pRb *in vivo* and *in vitro*, and mapped the HSP70-binding region of pRb [4,6]. In addition, it was shown that HSP 70 protected purified pRb from aggregation, thus protecting it from degradation *in vitro*. Based on these *in vitro* results, we investigated the functional significance of the HSP70-pRb interaction in cells.

In this study, we utilized a technology to transduce recombinant proteins into cells. A small domain consisting of 11 amino acids, termed TAT transduction domain, can direct a transduction of fusion proteins across cell membranes [15]. Transduced TAT fusion proteins could recover their proper functions in cells, even though they were purified in urea-denaturing conditions. The new method has already been applied to various cell biological analyses, in which proteins or synthetic peptides were efficiently transduced into a variety of cells and tissues. The advantage of this method resides in its simple and effective transduction of various recombinant proteins into almost all types of cultured cells even if proteins are fragile inside cells. Therefore this method can overcome the drawbacks of widely used methods such as microinjection and DNA transfection. We constructed bacterial expression vectors coding 6 histidine tag, T7 tag and 11

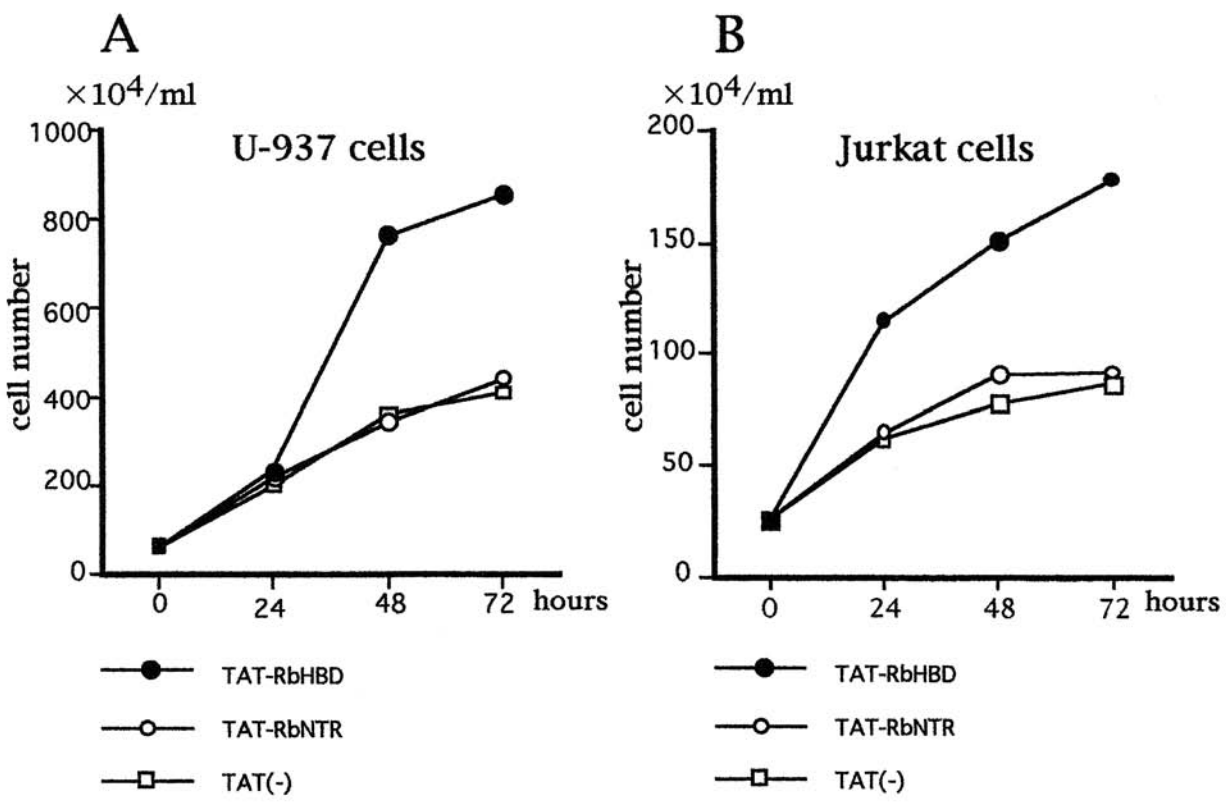

Fig.8 Effect of transduction of TAT fusion proteins on cell growth of Jurkat and U-937 cells

5 x 10^5 U-937 cells (panel A) or 2.5 x 10^5 Jurkat cells (panel B) were cultured in the absence (open square) or presence of 500 nM TAT-RbHBD (closed circle) or 500 nM TAT-RbNTR (open circle) protein at 37 ℃. Live cell numbers were counted every 24 h by trypan blue dye exclusion assay.

amino-acids TAT transduction domain, followed by multicloning sites derived from pET-21a vector, and subcloned in-flame PCR amplified DNA fragments coding either HSP-binding domain (RbHBD) or N-terminal region of human pRb (RbNTR). The resulting TAT fusion proteins were readily transduced into cultured cells by 2 h after addition into culture media. Transduced TAT-RbHBD protein, but not TAT-RbNTR protein, was shown to bind to HSP70 in cells and inhibit the association of HSP70 to endogenous pRb. Therefore, it was demonstrated that TAT-RbHBD protein could act as a competitive inhibitor to the HSP70-pRb association in cells.

We focused on the effect on cell growth, since pRb is an negative regulator of the cell cycle. Remarkably, transduction of TAT-RbHBD fusion protein accelerated cell growth of HOS cells, Jurkat cells and U-937 cells, which express functional pRb. In contrast, SAOS cells, which lacked pRb, were not affected by TAT-RbHBD protein. Therefore it was suggested that the growth acceleration by transduction of TAT-RbHBD protein could be mediated by pRb. In this context, we examined the effect of TAT-RbHBD protein on the protein level of dephosphorylated pRb in HOS cells. No obvious difference, however, was observed in the pRb levels between TAT-RbHBD-transduced cells and TAT-RbNTR-transduced cells (Fig. 6B). Since cells were treated with aphidicolin to increase the level of dephosphorylated pRb in this experiment, it is possible that the down-regulatory effect of TAT-RbHBD protein on the dephosphorylated pRb might have been masked by the effect of aphidicolin. Alternatively, transduction of TAT-RbHBD protein might have inhibited the HSP70-mediated conformational change of dephosphorylated pRb, leading to an increase of misfolded non-functional pRb.

The possibility that TAT-RbHBD protein also impaired the association of HSP70 to other substrate proteins in cells could not be ruled out in our study, since pRb is not the only target of HSP70 among proteins regulating the cell cycle. For example, tumor suppressor proteins p53 and WT1 also bind to HSP70 and require its chaperone function to suppress cell growth [19-21]. However, TAT-RbHBD protein was incapable of enhancing proliferation of pRb-deficient SAOS cells, indicating that the HSP70-pRb association might be selectively blocked by the TAT fusion protein.

Heat shock treatment reduced the effect of TAT-RbHBD protein on cell proliferation. Since such stress can dramatically induce the expression of HSP70 in cells, it is likely that the increased protein level of HSP70 canceled the competitive inhibitory action of TAT-RbHBD protein. We have previously examined heat-induced denaturation of purified pRb *in vitro*. Dephosphorylated pRb was very sensitive to heat stress, since purified pRb was aggregated by incubation at 42℃ for 30 min (data not shown). HSP70 and its co-chaperone HSP40 were capable of protecting dephosphorylated pRb from aggregation and degradation *in vitro*, suggesting that those molecular chaperones could indeed regulate the conformation of dephosphorylated pRb. It is known that a variety of stress, such as heat shock, can induce cell cycle arrest in certain cells. Under such stress, HSP70 and HSP40 translocate into the nucleus where most pRb resides. It is likely that the molecular chaperones bind to pRb and refold the denatured pRb, thus assisting the growth inhibitory function of pRb in cells under stress. It is noteworthy that proliferation of pRb-deficient SAOS cells was severely suppressed as compared to that of HOS cells after heat stress (Fig. 7). Our results indicate that there are other mechanisms for cell cycle regulation by stress proteins.

The role of HSP70 for potentiating the function of dephosphorylated pRb may also explain, at least in part, the mechanism for HSP70-mediated resistance against stress-induced cell death[22, 23, 24], since pRb is not only a growth suppressor but also an important factor for cell survival [8-11]. In this context, it is of interest to examine if there may be some cells for which dis-

ruption of the HSP70–pRb association leads to apoptosis.

Our study revealed that the association of HSP70 to pRb was very important for the growth control of cells expressing functional pRb and provided novel and important insights into the field of cell cycle regulation by stress proteins.

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