A Critical Role for the Proteasome Activator PA28 in the
Hsp90-dependent Protein Refolding*

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The 90-kDa heat shock protein, Hsp90, was previously shown to capture firefly luciferase during thermal inactivation and prevent it from undergoing an irreversible off-pathway aggregation, thereby maintaining it in a folding-competent state. While Hsp90 by itself was not sufficient to refold the denatured luciferase, addition of rabbit reticulocyte lysate remarkably restored the luciferase activity. Here we demonstrate that Hsc70, Hsp40, and the 20 S proteasome activator PA28 are the effective components in reticulocyte lysate. Purified Hsc70, Hsp40, and PA28 were necessary and sufficient to fully reconstitute Hsp90-initiated refolding. Kinetics of substrate binding support the idea that PA28 acts as the molecular link between the Hsp90-dependent capture of unfolded proteins and the Hsc70- and ATP-dependent refolding process.

Multiple interactions between distinct molecular chaperones and their cofactors are essential to accomplish proper protein (re)folding in the cell (1–6). While the ATP-dependent reaction mechanisms of binding and release of substrate proteins by Hsp70 and chaperonin families are well characterized (1, 4, 6), far less is known about the role of Hsp90, another major molecular chaperone in the eukaryotic cytosol (7). In reconstituted systems, Hsp90 prevents protein aggregation (8) and assists protein (re)folding (9, 10) in an ATP-independent manner. However, a requirement for ATP was described for the in vivo function of yeast Hsp90 (11, 12). Recently Rutherford and Lindquist (13) proposed that Hsp90 normally suppresses the mechanisms of binding and release of substrate proteins by Hsp90 was able to maintain thermally denatured luciferase against acute thermal damages, prior to induction of heat-shock proteins, which include Hsp90 itself (14). However, the molecular events linking Hsp90-initiated prevention of protein aggregation and its final refolding of the substrate proteins remained unclear. Here we demonstrate a new role of Hsc70 and Hsp40, a mammalian cytosolic homolog of DnaJ, in the reactivation process of thermally denatured luciferase. Freeman and Morimoto (16) first suggested that Hsp90 was able to maintain β-galactosidase in a refoldable state and furthermore, they showed that both Hsc70 and Hdj1(Hsp40) directly mediated its refolding to the native state under their experimental conditions. However, in our hands, using luciferase as a substrate, we determined that despite the presence of both Hsc70 and Hsp40, its reactivation still required a fraction of RL. This suggested that a third component in the RL may also be necessary for full accomplishment of the refolding process. We here report the identification of PA28, an activator of the 20 S proteasome, as a required cofactor working in concert with Hsc70 and Hsp40 during substrate refolding.

Previously, PA28 had been characterized as a component which enhanced the peptidase activity of the 20 S proteasome in an ATP-independent manner, without directly affecting the degradation of proteins or of ubiquitin-protein conjugates (17, 18). PA28 is a hexameric ring-shaped complex composed of two subunits, PA28α and PA28β (19), while recombinant PA28 forms a heptamer (20, 21). In vivo, a role of PA28 in antigen processing has been suggested (22, 23), although the biological significance of this complex remains largely enigmatic (24, 25).

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§§The abbreviations used are: RL, reticulocyte lysate; MOPS, 3-(N-morpholino)propanesulfonic acid; DTT, dithiothreitol; BSA, bovine serum albumin; Sulfo-NHS, sulfonated N-ethyl-3-dithiopropionate; Sulfo-NHS, sulfonated N-hydroxysuccinimido; PAGE, polyacrylamide gel electrophoresis.
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Since no homolog of PA28 has been found in yeast, PA28 may have a specific function in higher eukaryotes (25). Here our results suggest that PA28 not only serves as an activator of the peptidase activity of 20 S proteasome as shown previously, but also plays a critical role as a cofactor which functions in concert with Hsc70 and Hsp40 during the Hsp90-dependent protein refolding.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Porcine Hsp90, bovine Hsc70, and recombinant human Hsp40 were purified as described (26, 27). PA28 was purified from bovine blood cells kindly provided by Kawasumi Laboratories, Inc. (Oita, Japan), as described (17, 28) with the following modifications. The Sephacyr S-300 (Amersham Pharmacia Biotech) fractions were subjected to Q-Sepharose (Amersham Pharmacia Biotech) column purification. The PA28-containing fractions were combined with the purified 20 S proteasome and concentrated by ultrafiltration followed by desalting through a PD-10 column (Amersham Pharmacia Biochem). This partially purified PA28-20 S proteasome complex was further subjected to hydroxyapatite (Bio-Gel HTTP; Bio-Rad) chromatography to separate PA28 from the 20 S proteasome, followed by a second Sephacyr S-300 chromatography. Bovine 20 S proteasome was purified as described (29).

**Thermal Inactivation and Refolding of Luciferase**—Firefly luciferase (Sigma) was thermally inactivated for 5 min at 45 °C or 10 min at 42 °C at a final concentration of 0.26 μM in 30 mM MOPS-KOH, pH 7.2, 2 mM dithiothreitol (DTT) solution containing either Hsp90 or bovine serum albumin (BSA) as indicated. For refolding assays, thermally inactivated luciferase was diluted 10-fold into refolding buffer (10 mM MOPS-KOH, pH 7.2, 50 mM KCl, 3 mM MgCl₂, 2 mM DTT) containing the indicated components. Rabbit RL was obtained from Promega. The approximate concentrations of chaperones in RL are reported to be: Hsp90, 2 μM; Hsc70, 2–4.2 μM; Hsp40, 0.15 μM (30, 31). Aliquots (2 μL) were withdrawn from the reaction mixtures after incubation for 30 min, 1, or 1.5 h as indicated at 30 °C and mixed with a Promega luciferase assay system (40 μL). Luciferase activities were immediately measured by a Bio-Orbit 1253 luminometer and expressed as the percentage of the activity before thermal inactivation.

**Cross-linking**—The heterobifunctional cross-linker 2-[6-(biotinamido)-2-(p-azidobenzamido)-hexaamidoethyl]-1,3-dithiopropionate (Sulfo-SBED) (Pierce) was attached to luciferase via sulfonated N-hydroxysuccinimimidio (Sulfo-NHS) ester. The modified luciferase (−0.2 μM) was thermally inactivated in the presence of Hsp90 (11 μM) and diluted into refolding buffer (2 mM DTT) containing the indicated components to initiate refolding as described above. After photolysis at 312 nm for 5 min, the reaction mixtures were incubated for 30 min with 50 mM DTT to allow transfer of biotin-tag. The reaction mixtures were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Biotinylated proteins were visualized with horseradish peroxidase-streptavidin and chemiluminescence reagents (NEN Life Science Products Inc.) after transfer to a polyvinylidene difluoride membrane (Millipore).

**Light Scattering Assay**—Luciferase (0.1 μM) was mixed with either Hsp90 or BSA as indicated in refolding buffer. Aggregation was monitored by measuring the turbidity of the mixtures at 320 nm for 20 min at 45 °C by a Amersham Pharmacia Biotech Ultrospec 3000 UV/Vis spectrophotometer equipped with a thermostat holder.

**Proteinase K Resistance of Luciferase**—Luciferase (0.26 μM) was mixed in a refolding buffer with Hsp90 (12 μM) or BSA (12 μM), and incubated for 5 min at 45 °C. As a control, luciferase alone was incubated in the same way and then Hsp90 (12 μM) was added to the reaction. Proteinase K (Stratagene) treatment was performed for 10 min at 0 °C at 0–10 μg/ml and was stopped by adding phenylmethylsulfonyl fluoride to 2 mM (32, 33). The reaction mixtures were analyzed by SDS-PAGE and immunoblotting using anti-luciferase antibodies (BioGenes).

**Identification of the 30-kDa Protein**—Rabbit RL was diluted with a buffer solution (HEPES-KOH, pH 7.5, 0.1 mM EDTA) and ultracentrifuged to remove ribosomes. For reactivation assay, samples were concentrated 2–5-fold with Microcon-10 (Amicon) and mixed with Hsc70 (5.3 μM) and Hsp40 (2.2 μM) in refolding buffer containing ATP (3 mM). Finally, partially denatured luciferase preincubated in the presence of Hsp90 was added and mixed. Activities were measured at 30 min or 1 h. RL was eluted to a DEAE-cellulose (DE-52; Whatman) column and eluted with a linear gradient of 0–500 mM KC1 in the same buffer. Fractions containing the activity were pooled and subjected sequentially to 40, 60, and 80% saturation of ammonium sulfate. The 40–60% ammonium sulfate fraction that contained activity was applied to a Sephacryl S-300 column. A peak activity was eluted on a hydroxyapatite column with a linear gradient of 10–250 mM potassium phosphate (pH 7.5). Finally, pooled fractions from hydroxyapatite were diluted and applied to a Q-Sepharose column, followed by elution with a gradient of 100–500 mM KC1 in 10 mM HEPES-KOH, pH 7.5. After Q-Sepharose chromatography, the 30-kDa protein band was recovered from Coomassie-stained polyacrylamide gels and subjected to digestion with lysylendopeptidase (Wako, Japan). Recovered peptide fragments were sequenced as described (34). Appropriate fractions in each step described above were subjected to SDS-PAGE and immunoblotting using anti-peptide antibodies against human Hsp28α and PA28β (35).

**Immunodepletion of PA28**—Rabbit polyclonal antibodies were raised against histidine-tagged recombinant human PA28α (28). Hemoglobin A1C was removed from RL by passing through a column chromatography to yield "fraction II" (36). Fraction II equivalent to 20 μl of original RL was mixed with 20 μl of antibodies cross-linked to Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) for 1 h at 4 °C for immunodepletion. The supernatant after spinning down the beads was subjected to the second round of immunodepletion. Immunodepletion was assayed by Western blot analysis.

**Reconstitution of Recombinant Mouse PA28**—Mouse cDNA clones corresponding to PA28α and PA28β (37) were digested with NcoI and EcoRI, or NdeI and EcoRI, respectively. Each cDNA fragment encompassing an entire coding region starting at the initiation codon, was inserted into pET23d (digested with NcoI and EcoRI) or pET23a (digested with NdeI and EcoRI), respectively. Recombinant proteins were expressed as described (27) and purified according to the method described for purification of bovine PA28. Hetero-oligomers were reconstituted from purified PA28α and PA28β as described (38).

**Chemical Inactivation and Refolding of Luciferase**—Luciferase (10 μM) was denatured at room temperature by incubation for 30 min in a buffer containing 6 mM guanidinium-HCl, 30 mM MOPS-KOH, pH 7.2, and 2 mM DTT. Denatured luciferase was diluted 100-fold (0.1 μM) final concentration) with a refolding buffer containing 3 mM ATP, an ATP regenerating system (10 mM creatine phosphate and 50 μg/ml creatine phosphokinase) and the indicated components (Hsc70, 4 μM; Hsp90, 3 μM; reconstituted PA28, 15 μM; BSA, 15 μM). The reaction mixtures were incubated for 1 h at 30 °C and luciferase activities were measured as described above.

**RESULTS**

**The Capture of Luciferase by Hsp90**—Heat induction of the chaperone activity of Hsp90 and the initiation of the refolding process could be readily monitored by stabilizing the capture by Hsp90 dimer (39, 40) of a substrate, the firefly luciferase, to which a photoreactive biotin-tag was conjugated via Sulfo-NHS ester, using an ultraviolet (UV)-induced cross-linker. The biotin-tag was transferred to Hsp90 when UV was irradiated and the disulfide bond was cleaved with DTT (Fig. 1, A and 2). Following the capture of the luciferase by Hsp90, while a significant amount of biotin-tag was covalently trapped on the luciferase molecule itself, presumably through intramolecular cross-linking. In the absence of UV irradiation, no intermolecular tag transfer to Hsp90 was detected and the intramolecular tagging to luciferase was limited (Fig. 1A, compare lanes 1 and 2).

When Hsp90 and luciferase were separately heated, and then combined at room temperature, the tag was not transferred from luciferase to Hsp90 upon UV exposure (Fig. 1A, compare lanes 3 and 4). Thus the tag transfer reflected the capture of luciferase by Hsp90 that occurred during heat inactivation. Heat-induced luciferase association was further investigated by measuring the effect of Hsp90 on the rise in turbidity resulting from the aggregation of luciferase (Fig. 1B). During heat, Hsp90 suppressed luciferase aggregation in a concentration-dependent manner, while BSA had no effect on the initial velocity of aggregation and even increased the total turbidity. These data, taken together, support the idea that Hsp90 possesses a heat-inducible chaperone activity toward luciferase in vitro.

Next we assayed the protease susceptibility of luciferase to...
In Vitro Reconstitution of Hsp90-dependent Protein Refolding of Luciferase—Despite capturing of the luciferase by Hsp90 during heat inactivation, the activity of luciferase was completely lost at the end of the 5-min incubation at either 45 or 50 °C (data not shown), suggesting that the Hsp90-initiated reorganization was not able to fully refold luciferase. However, addition of 50% RL after the thermal inactivation resulted in a dramatic reactivation, in an Hsp90 concentration-dependent manner (Fig. 2A). A 10-fold excess amount of Hsp90 dimer present during the heat inactivation process was largely sufficient to enable RL-dependent reactivation of thermally denatured luciferase, while BSA could not substitute for Hsp90 (Fig. 2A). Although the capture of denatured luciferase by Hsp90 was also apparent upon a milder heat treatment at 42 °C (Fig. 2B), the potency of Hsp90 to maintain luciferase in such a refolding-competent state tended to be more manifested at higher temperatures (Fig. 2A), in line with an increased transfer of biotin-tag at 50 °C compared with 45 °C (Fig. 1A, lanes 7 and 8). Hereafter we choose the condition of 45 °C for 5 min in order to emphasize the dependence on Hsp90 of the refolding of thermally denatured luciferase. Our finding that Hsp90 maintained partially unfolded luciferase in a folding-competent state is consistent with the finding by Freeman and Morimoto (16). Thus Hsp90 may play an important role in the protection of the cytosolic proteins against heat damages.

Reducing the amount of RL added during the incubation following heat inactivation from 50% to less than 5% provided a way to examine the requirement for various cofactors during the refolding process. When purified Hsc70 and Hsp40 were supplemented in the presence of 3% RL and ATP, a full refolding activity comparable to the effect of 50% RL was reconstituted (Fig. 2C). However, the exclusion of either one of the four components (RL, Hsc70, Hsp40, and ATP) abolished the refolding activity. These results indicated that Hsc70 indeed participated in the refolding reaction of luciferase, and that Hsp40 and at least another additional component in the RL may also be necessary for full accomplishment of the refolding/reactivation process.

To gain insight into the molecular proximity between the various protein factors involved in the refolding process, we examined the extent of tag transfer to each one of the necessary components. Upon refolding of luciferase by addition of Hsc70, Hsp40, and RL, biotin-tag was immediately transferred to Hsp90 (Fig. 2D). The tag appeared to be also transferred to Hsp40, although only to a much smaller extent. These data suggested that the luciferase, initially bound to Hsp90, may be released and bind to other factors, and that Hsc70, Hsp40, and a third yet unidentified factor present in RL, may cooperate in this process. Recently several cofactors of Hsc70 and Hsp90, such as Hip/p48, p60/Hop, and p23, have been documented (3, 5); these may include a candidate for the unknown factor. In our hands, purified Hip (41) could not complement the lack of RL during the reactivation of luciferase even in the presence of both Hsc70 and Hsp40 (data not shown). The reported elution positions for Hop and p23 on a DEAE-cellulose column (42) also seemed to differ from those of the reactivation activities eluted with a DEAE-cellulose column (see below). Taken together, these data suggested that RL contained a necessary cofactor distinct from Hsp90, Hsc70, Hsp40, or known cofactors such as Hip, Hop, or p23, and that this cofactor was critical in the reactivation of thermally denatured luciferase.

Identification of PA28 as the Cofactor—To elucidate the molecular identity of this factor, we attempted to purify the active probe for a potential change in its structural flexibility in the presence or absence of Hsp90. The conformation of chaperone-bound protein substrate is in a collapsed state with a high protease sensitivity and in contrast, the heat-aggregated substrate is highly resistant to protease (32, 33). Thermal denaturation in the presence of Hsp90 substantially increased the protease K sensitivity of luciferase, while addition of BSA had no such effect (Fig. 1C). Thus, binding of heat-activated Hsp90 not only prevented aggregation but also initiated a reorganization in the tertiary structure of the luciferase, as indicated by the increase in protease sensitivity.
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**Fig. 2.** Hsp90-dependent refolding of luciferase. A, luciferase (0.26 μM) was inactivated for 5 min at 45 or 50 °C in the presence of Hsp90 or BSA as indicated. Luciferase activities were measured after a 1-h incubation with or without 50% RL (mean ± S.E.; n = 3). B, thermal inactivation of luciferase (0.26 μM) was performed for 10 min at 42 °C in the presence of Hsp90 (2.9 μM) or BSA (2.9 μM). Luciferase activities were measured after 30 min incubation with or without 50% RL. C, luciferase (0.26 μM) was inactivated for 5 min at 45 °C in the presence of Hsp90 (11 μM). Reactivation was performed for 1 h with the indicated combinations of RL, Hsc70 (3.9 μM), Hsp40 (2.2 μM), and ATP (3 mM). D, biotin-tagged luciferase (~0.2 μM) was incubated for 5 min at 45 °C in the presence of Hsp90 (11 μM) and refolding was carried out by incubation with RL (5%), Hsc70 (4.2 μM), and Hsp40 (2.2 μM) for 0, 10, 20, and 40 min (lanes 1–4) at 30 °C. At the end of each incubation, a cross-link was induced by UV irradiation to detect components interacting with luciferase at that instant. 70, Hsc70; 40, Hsp40. Asterisks indicate unidentified protein bands included in RL, nonspecifically reacting with peroxidase-streptavidin, since they were visible even in the control devoid of biotin-tagged luciferase (data not shown).

component(s) (referred hereafter as cofactor activity) contained in the RL, that enables the reactivation of luciferase captured by Hsp90 during thermal inactivation in the presence of Hsc70 and Hsp40. A single broad peak was eluted from a DEAE-cellulose column at around 250 mM KCl (data not shown), further concentrated by ammonium sulfate fractionation, and then applied to a Sephacryl S-300 size-exclusion column. The cofactor activity was recovered at a relative molecular mass of 160 kDa (data not shown). This fraction was further chromatographed on hydroxyapatite and Q-Sepharose columns. Although the fractions containing the peak activity at this stage still included more than 10 proteins (Fig. 3), we found that elution of a protein band of 30 kDa most closely coincided with the cofactor activity enriched through the above steps (data not shown).

This 30-kDa protein was separated and isolated as two close bands on a preparative SDS-PAGE. While the N-terminal amino acid sequences for both were blocked and could not be determined, sequences obtained from lysylendopeptidase-digested peptide fragments revealed that the two bands corresponded to the proteasome activator PA28a and PA28β, respectively (35) (Fig. 3). Immunoblot analyses using an anti-peptide antibody against the human PA28α showed that PA28α was co-eluted with the cofactor activity at each purification step (data not shown). A similar result was obtained by using an anti-peptide antibody against human PA28β (data not shown). These results raised the possibility that PA28α and PA28β may participate as cofactors in the reactivation of thermally denatured luciferase. An apparent discrepancy was observed between the molecular size of this factor on an SDS-PAGE (30 kDa; Fig. 3) and that on a size-exclusion column (160 kDa); however, this is consistent with previous reports showing that PA28 probably exists as a heterohexamer (19).

Requirement of PA28 for the Reactivation of Luciferase—To establish the role of PA28 in the reactivation of thermally denatured luciferase, we carried out immunodepletion and reconstitution experiments, using PA28 purified to homogeneity from bovine blood cells (Fig. 4A). First, we investigated whether PA28 was truly a part of the required component(s). It was difficult to immunodeplete PA28 directly from RL, because of the presence of a large amount of hemoglobin. To circumvent this difficulty, hemoglobin was removed from RL by passing it through DEAE-cellulose column chromatography to yield fraction II, a high salt eluate containing most of the non-hemoglobin proteins (36). The cofactor activity was totally absent from the flow-through fraction, while it was almost fully recovered in fraction II (data not shown). When fraction II was immunodepleted using an anti-PA28 antibody, a significant reduction in the cofactor activity was observed (Fig. 4B, columns 1 and 2), in parallel with the removal of the majority of PA28 immunoreactivity (Fig. 4B, inset). To ascertain that the reduction in the cofactor activity was mediated by the exclusion of PA28 and not due to an indirect effect, we examined whether readdition of PA28 could complement the loss of cofactor activity in PA28 immunodepleted fraction II. Indeed this was the case, since the decreased activity was restored to its full potency in the presence of purified PA28 (Fig. 4B, column 3). These data strongly argue that PA28 participates in and may be necessary for the reactivation of thermally denatured luciferase.

We next asked whether PA28 was sufficient by itself to provide a full cofactor activity. When the activity of purified bovine PA28 (Fig. 4A) was measured in the presence of Hsc70 and Hsp40, PA28 enhanced the reactivation of luciferase to an extent similar to that provided by RL in previous experiments (Table I and Fig. 2C). To confirm this result, the reactivation of luciferase was examined using the reconstituted PA28 (Table
lysylendopeptidase-digested fragments for each band (7-1, 7-2, 9, and Q-Sepharose). The 30-kDa protein band, upon more detailed examination, consisted of a doublet (data not shown). Internal sequences of parentheses sequence identity (in shown together with the corresponding deduced amino acid sequences from human PA28 (35) with amino acid numbers (of biotin-tagged luciferase bound to Hsp90, after addition of the we analyzed the transfer of the biotin-tag during the refolding we confirmed that the tag was transferred to Hsc70 with a % of control 20 73 0.8 5.5 0.5
The cofactor activity of the reconstituted mouse PA28 Reactivation of luciferase (0.26 \( \mu \text{M} \)) which was heat-denatured in the presence of Hsp90 (11 \( \mu \text{M} \)), was performed by incubation for 1 h with the indicated concentrations of the reconstituted mouse PA28 in the presence of ATP (3 \( \mu \text{M} \)), and an ATP-regenerating system (10 mM creatine phosphate and 50 \( \mu \text{M} \) creatine phosphokinase). An ATP-regenerating system, which alone reactivated thermally denatured luciferase by only 0.1%, was required for the refolding reaction with the reconstituted PA28, although it is not interpretable yet.

| PA28 | Hsc70 | Hsp40 | Luciferase activity % of control |
|------|-------|-------|---------------------------------|
| 12   | 2     | 0.5   | 5.5                             |
| 11   | 2     | 0.8   | 0.5                             |
| 21   | 1     | 20    | 20                             |

The Role for PA28 in the Reactivation of Luciferase—To further elucidate the role of PA28 in the reactivation process, we analyzed the transfer of the biotin-tag during the refolding of biotin-tagged luciferase bound to Hsp90, after addition of the three cofactors, Hsc70, Hsp40, and purified bovine PA28. First, we confirmed that the tag was transferred to Hsc70 with a coincident decrease in the amount of biotin-tag transferred to Hsp90, at a rate similar to that observed in Fig. 2D, again confirming that purified PA28 could fully replace RL (Fig. 5). More interestingly, PA28 also received the biotin-tag immediately after the onset of refolding (Fig. 5), indicating that PA28 itself is implicated in the early events of the refolding process, probably through a direct interaction with the substrate. Furthermore, the rise in the amount of biotin-tagged PA28 sharply mirrored the decline in biotin-tag binding to Hsp90, with the peak of the biotin-tag transfer to PA28 preceding that seen for Hsc70 (Fig. 5). This raised the possibility that PA28 may provide an initial, transitory binding site for the substrate, during the transfer of denatured luciferase from Hsp90 to Hsc70.
Consistent with this idea, we found that the amount of biotin-tag transferred to PA28 slowly decayed with time, while the biotin-tag bound to Hsc70 remained elevated (Fig. 5).

We examined whether PA28 affects the refolding of chemically denatured luciferase (Table III). Neither Hsc70 and Hsp40 nor PA28 alone mediated efficient refolding of denatured luciferase. However, a combination of PA28 and Hsc70/Hsp40 enabled a remarkable reactivation of luciferase. BSA could not stimulate the luciferase refolding even in the presence of Hsc70 and Hsp40. PA28 had no effect upon the ATPase activity of Hsc70 (data not shown). These results suggested that the role of PA28 in protein folding as a molecular chaperone may not be restricted to the Hsp90-mediated protein refolding process.

**DISCUSSION**

Based upon our results, we here suggest the following model for the involvement of PA28, Hsc70, and Hsp40 during the refolding of luciferase bound to Hsp90. Upon heat, not only is luciferase inactivated and denatured, but also Hsp90 gains higher ability to bind to its substrate, leading to a tight complex formation between the Hsp90 and unfolded luciferase. After the luciferase is captured by Hsp90, PA28 immediately associates with this complex, which in turn is followed by the dissociation of Hsp90 from the substrate. Hsc70 then displaces PA28, thereby leading to the refolding of the substrate. In this step, Hsp40 is likely to function as a cofactor of Hsc70. Finally, correct refolding of luciferase is obtained, as Hsc70 and Hsp40 are ultimately released. In this model, PA28 serves as a capturing protein that binds the partially unfolded luciferase and maintains it in a refolding-competent state, while the recruited PA28 serves as a transient adaptor, pushing the substrate from an Hsp90-bound to an Hsc70-bound state. Freeman and Moriguchi (16) reported that Hsc70 and Hsp40 directly mediate the refolding of β-galactosidase chemically denatured and bound to Hsp90 at 37 °C. In contrast, in our hands using heat-inactivated luciferase as a substrate, induction of the chaperone activity of Hsp90 required a higher temperature (14), and luciferase was presumably more severely unfolded and more tightly bound to Hsp90. The present work provides compelling evidence that under these conditions, PA28 is a required cofactor for the Hsp90-dependent protein refolding and reactivation, and likely acts as a molecular link between the chaperone activity of Hsp90 and the subsequent reactivation process mediated by several cofactors including Hsc70 and Hsp40.

Previously, PA28 had been characterized as a component which enhanced the peptidase activity of the 20 S proteasome (24, 25). However, since free peptides are rare in cells, the physiological relevance of the complex between PA28 and the 20 S proteasome remains elusive (25). Here, we found that PA28 acts as a dual regulator of protein remodeling and degradation in vivo? Although specific test of this hypothesis requires further work, it is worthwhile to consider a reminiscent case in the prokaryotic system. In *Escherichia coli*, ClpA and ClpX were shown to participate in protein remodeling as molecular chaperones, while they also acted as regulatory subunits of ATP-dependent protease, ClpP and ClpXP, respectively, involved in protein degradation (43). Quite recently, chaperone activity of another proteasome activator PA700 was suggested (44, 45). Schneider et al. (46) reported that misfolded luciferase bound to Hsp90 was degraded through the ubiquitin-proteasome pathway, in which the 20 S proteasome formed the 26 S proteasome complex with PA700 but not with PA28 (24, 25). Another study, however, suggested that the 20 S proteasome could simultaneously bind both PA28 and PA700 (47). Thus, under conditions where luciferase could not be successfully transferred from PA28 to Hsc70 for its full reactivation, PA28 may reroute the substrate binding to PA700 but not with PA28 (24, 25). Our current work further strengthens this hypothesis and further investigation is under way to elucidate the role for the PA28 dual activity in regulating the Hsp90-dependent stringent quality control of protein folding within the eukaryotic cytosol.

**TABLE III**

Reactivation of chemically denatured luciferase

| Hsp70/Hsp40 | PA28 | BSA | Luciferase activity | % of control |
|-------------|------|-----|---------------------|-------------|
| +           | +    | −   | −                   | 13          |
| +           | +    | −   | −                   | 52          |
| −           | +    | −   | −                   | 13          |
| +           | −    | +   | −                   | 5.3         |

Fig. 5. Transfer of the biotin-tag from luciferase into Hsp90, PA28, and Hsc70. Biotin-tagged luciferase (0.2 μM) was incubated for 5 min at 45 °C in the presence of Hsp90 (11 μM). Refolding was carried out in the presence of purified bovine PA28 (2 μM), Hsc70 (4.2 μM), and Hsp40 (2.6 μM) for 0, 8, 15, and 30 min (lanes 1–4) at 37 °C.
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