Archives of Biochemistry and Biophysics
Vol. 384, No. 1, December 1, pp. 59–67, 2000
doi:10.1006/abbi.2000.2075, available online at http://www.idealibrary.com on IDEAL®

Reactive Cysteines of the 90-kDa Heat Shock Protein, Hsp90

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Received April 27, 2000, and in revised form August 30, 2000

The 90-kDa heat shock protein (Hsp90) is the most abundant molecular chaperone of the eukaryotic cytoplasm. Its cysteine groups participate in the interactions of Hsp90 with the heme-regulated eIF-2α kinase and molybdate, a stabilizer of Hsp90–protein complexes. In our present studies we investigated the reactivity of the sulfhydryl groups of Hsp90. Our data indicate that Hsp90 as well as two Hsp90 peptides containing Cys-521 and Cys-589/590 are able to reduce cytochrome c. The effect of Hsp90 can be blocked by sulfhydryl reagents including arsenite and cadmium, which indicates the involvement of the vicinal cysteines Cys589/590 in the reduction of cytochrome c. Hsp90 neither reduces the disulfide bonds of insulin nor possesses a NADPH:quinone oxidoreductase activity. Oxidizing conditions impair the chaperone activity of Hsp90 toward citrate synthase. The high and specific reactivity of Hsp90 cysteine groups toward cytochrome c may indicate a role of this chaperone in modulation of the redox status of the cytosol in resting and apoptotic cells.

Key Words: **apoptosis; cytochrome c; disulfide bonds; Hsp90; molecular chaperone; sulfhydryl groups.**

Several molecular chaperones are actively involved in the regulation of the redox status of other proteins by assisting in the formation and breakage of disulfide bridges (1, 2). Chaperones help to achieve and maintain the conformational homeostasis of cellular proteins and probably played a major role in the development of modern enzymes (3, 4). The 90-kDa heat shock protein (Hsp90)[3] is an abundant organizer of the cytosolic chaperone complex (the "foldosome") helping *de novo* synthesized nuclear hormone receptors and several protein kinases to reach and preserve their unstable conformation in an "activation-competent" state (5–7). Its cysteine groups were thought to participate in the interactions of Hsp90 with heme-regulated eIF-2α kinase (8). Our recent findings (9) indicated an involvement of Hsp90 cysteines in binding of molybdate, a stabilizer of Hsp90–target interactions (6, 7, 10).

Protein disulfide isomerases are well-known examples of the simultaneous appearance and utilization of redox and chaperone properties of the same protein (1, 2). However, an increasing number of studies indicates that other, unrelated molecular chaperones may also possess various enzymatic activities changing the redox status of another protein or small substrate. Thus it has been reported that the cysteine in the YSCVGVF sequence of Hsp70 is able to reduce cytochrome c (11). Quite unexpectedly, the cochaperone of bacterial Hsp70, the DnaJ protein, turned to be a protein disulfide isomerase by itself (12). Another chaperone, ζ-crystallin, was shown to possess a NADPH:quinone oxidoreductase activity (13). Recently, Hsp33, a member of a newly discovered chaperone family was shown to possess a chaperone activity only after its sulfhydryl groups were oxidized to disulfide bridges (14).

Encouraged by the multiple appearance of potent redox activities in a variety of molecular chaperones in the present report we have studied the reactivity of cysteine-containing sequences of Hsp90 analyzing their ability to reduce cytochrome c, to promote protein

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[3] Abbreviations used: DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GSSG, oxidized glutathion; Hsp70, 70-kDa heat shock protein; Hsp90, 90-kDa heat shock protein.

0003-9861/00 $35.00

disulfide isomerization and to participate in the chaperone activity of Hsp90. The ability of Hsp90 to reduce cytochrome c and the high redox reactivity of its cysteines 521 and 589/590 provides another example, where a molecular chaperone is able to facilitate redox reactions and may indicate a role of Hsp90 in maintenance of the redox status of the cytosol. Oxidizing conditions impair the chaperone activity of Hsp90 toward citrate synthase, which is a further proof for the active participation of sulfhydryl groups in the function of Hsp90.

MATERIALS AND METHODS

Chemicals. Peptides were synthesized on 2-chlorotrityl resin as described earlier (15) using the combination of BOC and FMOC technique. The cysteine SH was protected by tertiary butyl group. After cleavage from the resin with a mixture of dichoromethane: acetic acid:trifluoroethanol (4:1:1), the protecting groups were removed by HF in the presence of 10% anisole at −5°C. Finally the compound was purified by reversed-phase HPLC using acetonitrile–water–(0.1% trifluoroacetic acid) mixture. The homogeneity of the compound was checked by analytical HPLC. FAB mass spectrometry of the purified product yielded the expected molecule-ion.

Fluoresceine isothiocyanate (FITC)-labeled insulin was a kind gift from Drs. Alejandro P. Heuck and Ricardo A. Wolosiuk (Instituto de Investigaciones Bioquimicas, Universidad de Buenos Aires, Buenos Aires, Argentina) (16, 17). DsbA and thioredoxin were purchased from Epicentre Technologies (Madison, WI) and from Promega (Madison, WI), respectively. Bio-Scale Q FPLC and Econo-Pac HTP cartridges were from Bio-Rad (Richmond, CA). Butyl-Sepharose 4B and DEAE-Sepharose Fast Flow were purchased from Pharmacia (Uppsala, Sweden). All other chemicals used were from Sigma Chemicals Co. (St. Louis, MO).

Purification of Hsp90. Hsp90 was purified from rat liver with consecutive chromatographies on Butyl-Sepharose 4B, DEAE-Sepharose Fast Flow, Econo-Pac HTP, and Bio-Scale Q FPLC columns as described earlier (9, 18). The purity of these Hsp90 preparations was more than 95% as judged by silver staining of SDS–polyacrylamide gels. Protein concentration was determined according to Bradford (19).

Reduction of cytochrome c. Reduction of cytochrome c was monitored by measuring the increase in the absorbance of the 550-nm band of cytochrome c (11). In a typical experiment 10 μM cytochrome c was added to a buffer containing 100 mM Hepes, pH 7.4, 100 mM NaCl. To cytochrome c, Hsp90 or Hsp90-derived peptides were added at the final concentrations indicated, and the reduction of cytochrome c was monitored in a final volume of 0.12 ml by measuring the change of its absorbance at 550 nm at 25°C using a GBC 920 spectrophotometer. The maximal absorbance of cytochrome c was obtained after the addition of a large excess of sodium dithionite. Absorbance values were corrected to the baseline at 600 nm and normalized to cytochrome c concentration obtained from the 550 absorbance of maximally reduced cytochrome c using the extinction coefficient of 21,000 (11).

Measurement of protein disulfide oxidoreductase activity. Protein disulfide oxidoreductase activity of Hsp90 and related peptides was measured by two separate methods monitoring the reduction of insulin disulfide bridges. In the "standard" assay the aggregation of insulin B-chains was monitored at 650 nm (with 1-nm slits) using a Hitachi F-4500 spectrofluorimeter as described by Holmgren (20). Insulin at a final concentration of 50 μM was added to a buffer containing 100 mM sodium phosphate, pH 6.5, 2 mM dithiothreitol, and 0.2 mM EDTA at a final concentration of 0.05 mM, and after the addition of Hsp90 or Hsp90-derived peptides at the final concentrations indicated, the aggregation of insulin B-chains was monitored at 25°C in a final volume of 0.2 ml.

In some experiments the assay of Wolosiuk and Heuck (16, 17) was also used to follow the separation of the A- and B-chains of FITC–insulin after the reduction of disulfide bridges. In this assay the buffer contained 100 mM sodium phosphate, pH 7.4, 3 mM EDTA, 0.1 mM dithiothreitol, and 0.7 μM FITC–insulin. Fluorescence of insulin-bound fluorescein was monitored at 25°C in a final volume of 0.2 ml using a Hitachi F-4500 spectrofluorimeter operated at the excitation/emission wavelength pair of 495/520 nm with 5 nm excitation and emission slits, respectively.

Measurement of sulfhydryl content. The amount of reduced sulfhydryl groups of Hsp90 and its peptides was determined by the Ellman method (21) using a molar extinction coefficient of 14,150 for 2-nitro-5-thiobenzoic acid (22).

Measurement of NADPH:quinone oxidoreductase activity. Measurement of NADPH:quinone oxidoreductase activity has been performed using the method of Rao *et al.* (13). Briefly, 1 μM purified Hsp90 or 5 μl postmitochondrial supernatant of rat liver homogenates containing 150 μg protein was added to 120 μl of reaction mixture containing 100 mM Tris–HCl, pH 7.8, 0.2 mM EDTA, 0.1 mM NADPH, and 20, 25, or 50 μM 1,2-naphthoquinone, 9,10-phenanthrenequinone, or juglone (5-hydroxy-1,4-naphthoquinone), respectively. Oxidation of NADPH (and in separate experiments, NADH) was monitored at 25°C by following its absorbance at 340 nm.

Measurement of chaperone activity. Pig heart citrate synthase (10 μM) (Boehringer) was incubated in denaturation buffer (50 mM Tris, 2 mM EDTA, 8 M urea, pH 8.0) for 1.5 h at room temperature. The renaturation was initiated by a 50-fold dilution in 40 mM Hepes/KOH, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM K acetate, pH 8.0, and 1.8 μM Hsp90. In some experiments the redox status of the renaturation buffer was changed by adding 1 mM ascorbic acid, or 1 mM dehydroascorbic acid, or 2.5 mM GSSG. The enzymatic activity of citrate synthase was measured as described by Jacob *et al.* (23).

RESULTS

Predicted Reactivity of the Hsp90 Sulfhydryl Groups

To assess the reactivity of various sulfhydryl groups of Hsp90 we have used the prediction method of Fiser *et al.* (24). Analyzing the neighboring 20 amino acids of more than 10,000 cysteines with documented covalent status they have established normalized abundance of amino acids in the vicinity of free cysteines and half-cystines. Using their method we have calculated the "disulfide bond forming potential" of the six cysteines of rat Hsp90 (25). The results of the prediction are presented in Table I. Among the six cysteines Cys-589 (amino acid sequence numbers refer to the rat Hsp90α sequence (25)) has an outstandingly high disulfide bond forming potential. Interestingly, the proline adjacent to this cysteine is the single residue among the 23 prolines of Hsp90, which is predicted to be in "cis" conformation using the prediction method of Frömmel and Preissner (26).

As another way to predict the ability of Hsp90 cysteines to reduce other compounds we compared the cysteine-containing sequences with the YSCVGVF sequence of Hsp70 known to reduce cytochrome c (11). The disulfide forming potential of the cysteine in the YSCVGVF sequence is 2.41, if calculated by the above method (24), which makes it almost as reductive as the

TABLE I

Disulfide Bond Forming Potential of Hsp90 Cysteine Groups[a]

Hsp90 sequence	Cys residue number	Disulfide bond forming potential
YVRRVFIMDS**C**DDLIPEYLNF	366	0.03
KVIRKNIVKK**C**LELFSELAED	412	0.02
VYMTEPIDEY**C**VQQLKEFDGK	521	0.16
EESKARFENL**C**KLMKEILDKK	564	0.06
TISNRLVSSP**C**CIVTSTYGWT	589	4.58
ISNRLVSSPC**C**IVTSTYGWTA	590	0.78

[a] The disulfide bond forming potential of Hsp90 cysteine groups was analyzed using the prediction method of Fiser *et al.* (23). The higher the disulfide bond forming potential of the given cysteine, the higher its ability to reduce other compounds. Amino acid sequence numbers refer to the rat Hsp90α sequence (24).

predicted value for Cys-589/590 of Hsp90. Peptide YCVQQL containing Cys-521 is highly similar to the YSCVGVF peptide, and the disulfide forming potential of Cys-521 is the third largest among the 6 cysteines of rat Hsp90.

Interestingly, the hydrophobic cluster analysis performed by Callebaut *et al.* (27) identified the regions of Cys-521 and Cys-589/590 as central parts of two putative mononucleotide binding units of the 90-kDa molecular chaperone family being highly similar to those of the NAD-binding domains of lactate-, alcohol-, and malate-dehydrogenases (27). Cys-521 lies in the second helix of the first putative $\beta\alpha\beta\alpha\beta$ mononucleotide binding unit, while Cys-589/590 are situated in the second beta strand of the second putative $\beta\alpha\beta\alpha\beta$ mononucleotide binding unit, respectively.

Reduction of Cytochrome c by Hsp90 and Its Peptides

Examining if Hsp90 is able to reduce cytochrome c we have observed a slow, but steady increase of the 550-nm peak in the absorption spectrum of cytochrome c, showing a gradual reduction of Fe^{3+} in the heme of cytochrome c (Fig. 1A). The absorption increase reached a half-maximal value around 10 min, began to level off after 30–40 min (Fig. 1B), and was not induced by bovine serum albumin, histone H3, globulin, or casein (Fig. 1B and data not shown).

Based on the fact that Hsp90 is an ATP-binding protein (18, 28) and on the observed interference between sulfhydryl group labeling and ATP-binding of Hsp70 (29) we have analyzed the effect of ATP on the Hsp90-induced cytochrome c reduction. Addition of 1 mM ATP did not cause a significant change in the cytochrome c reducing activity of Hsp90 (Fig. 1C). The reduction was unaffected by $CaCl_2$, $MgCl_2$, KCl, or NaCl at final concentrations of 5, 5, 150, and 150 mM, respectively (data not shown). On the contrary, addition of 1 mM *N*-ethylmaleimide caused an almost complete inhibition of cytochrome c reduction (Fig. 1C), which indicated that the reduction is mediated by the cysteines of Hsp90.

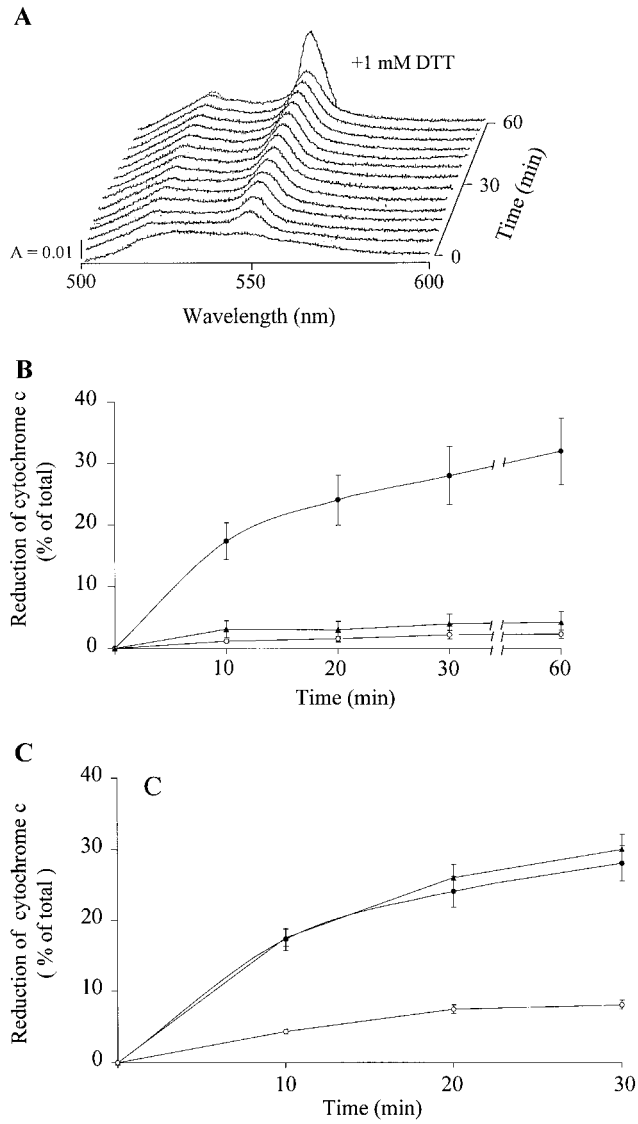

FIG. 1. Reduction of cytochrome c by Hsp90. Reduction of cytochrome c was monitored as described under Materials and Methods. (A) Changes of cytochrome c absorption spectrum after the addition of 0.9 μM Hsp90. DTT stands for dithiothreitol. Spectra are representatives of five independent experiments. (B) Time course of cytochrome c reduction. The 550-nm absorbance is shown after the addition of 1 μM Hsp90 (filled circles), bovine serum albumin (filled triangles), or buffer (open circles). Data are means ± SD of three independent experiments. (C) Effect of ATP and *N*-ethylmaleimide on Hsp90-induced cytochrome c reduction. The 550-nm absorbance is shown after the addition of 1 μM Hsp90 in the absence of added compounds (filled circles), and in the presence of 1 mM ATP and 2 mM $MgCl_2$ (filled triangles), or 1 mM *N*-ethylmaleimide (open circles). Data are means ± SD of three independent experiments. Total reduction of cytochrome c was achieved after addition of a large excess of sodium dithionite.

TABLE II

Sequences and Charges of Hsp90 Peptides Used in This Study

Name	Sequence	Net charge at neutral pH
Peptide I	YCVQQL	0
Peptide IIa	RLVSSPCCIVTSTYGW	+1
Peptide IIb	LVSSPCCIVTSTYGWTANME	−1
Peptide IIc	RLVSSPCCIVTSTYGWTANM	+1

Following the result of the prediction methods described above we have synthesized the two peptides containing the cysteines of Hsp90, Cys-521, and Cys-589/590 presumed to have the highest activity towards cytochrome c (peptides I and IIa of Table II). Both peptide I and peptide IIa were able to reduce cytochrome c (Fig. 2A). Peptide I was more effective than peptide IIa, since the amount of reduced cytochrome c was higher, and the concentration required for half-maximal reduction of cytochrome c was smaller in case of peptide I than in the case of peptide IIa (Fig. 2A). Peptide-induced reduction of cytochrome c was relatively rapid, reaching a half-maximal value after 2 to 10 min (Fig. 2B). Addition of 1 mM *N*-ethylmaleimide completely abolished the ability of both peptide I and IIa to reduce cytochrome c (data not shown). Charge variant peptides of peptide IIa (peptides IIb and IIc, Table II) did not show major differences in the reduction of cytochrome c (Fig. 2C). Comparison of the reductive efficiency of equimolar amounts (1 μM each) of Hsp90 peptides and Hsp90 revealed that peptide I, which had the highest efficiency to reduce cytochrome c among all peptides tested even at this small concentration, had the same reductive efficiency as Hsp90 itself (Fig. 2D).

Inhibition of Cytochrome c Reduction by Various SH-Reagents

Following our finding that the reduction of cytochrome c by both Hsp90 and its peptides can be inhib-

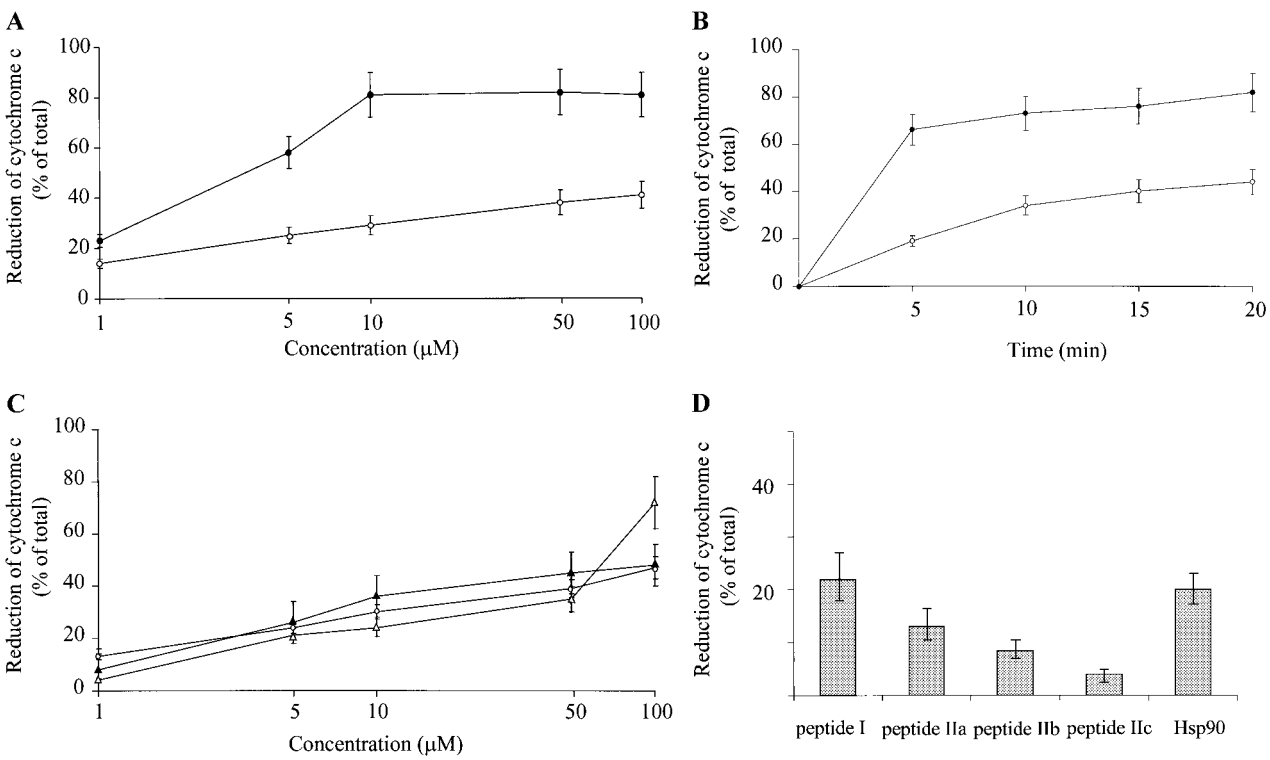

FIG. 2. Reduction of cytochrome c by Hsp90 peptides. Reduction of cytochrome c was monitored as described under Materials and Methods. (A) Concentration dependence of cytochrome c reduction. The 550-nm absorbance is shown 20 min after the addition of peptide I (filled circles) or peptide IIa (open circles). The sequences of the peptides are given in Table II. In the absence of peptides the reduction of cytochrome c was taken as 0%. (B) Time course of cytochrome c reduction. The 550-nm absorbance is shown after the addition of 100 μM peptide I (filled circles) or 100 μM peptide IIa (open circles). (C) Comparison of the concentration dependence of cytochrome c reduction induced by charge variants of peptide IIa. The 550-nm absorbance is shown 20 min after the addition of peptide IIa (open circles), peptide IIb (filled triangles), or peptide IIc (open triangles). In the absence of peptides the reduction of cytochrome c was taken as 0%. (D) Comparison of cytochrome c reduction by equimolar amounts (1 μM each) of Hsp90 and Hsp90 peptides. The sequences of the peptides are given in Table II. Data are means ± SD of three independent experiments. Total reduction of cytochrome c was achieved after addition of a large excess of sodium dithionite.

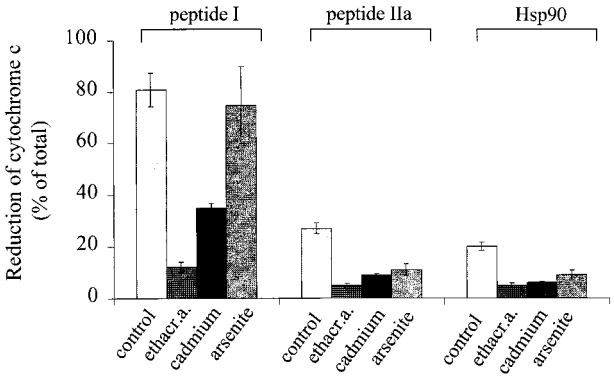

FIG. 3. Inhibition of Hsp90- and Hsp90 peptide-induced cytochrome c reduction by SH reagents. Reduction of cytochrome c was measured after 20 min as described under Materials and Methods using 1 μM Hsp90 or 10 μM each of peptide I and peptide IIa. Cytochrome c reduction was measured in the absence of SH reagents, or in the presence of 1 mM each of ethacrynic acid, $CdCl_2$, and arsenite as indicated. The sequences of the peptides are given in Table II. Data are means ± SD of three independent experiments.

ited by the SH-reagent, *N*-ethylmaleimide, we decided to study the effect of other SH reagents on cytochrome c inhibition in detail. We used 1 mM ethacrynic acid as a general SH reagent (30) and 1 mM $CdCl_2$ and 1 mM arsenite as specific reagents for vicinal thiols (31). The reductive effect of peptide I was not blocked by arsenite and was only partially affected by $CdCl_2$, which is in accordance with the absence of vicinal thiols in peptide I. On the contrary, peptide IIa-induced cytochrome c reduction was efficiently blocked both by arsenite and $CdCl_2$, which is in agreement with the presence of a vicinal thiol-pair in peptide IIa. Cytochrome c reduction in the presence of Hsp90 was also significantly inhibited both by arsenite and $CdCl_2$, which indicates the involvement of Cys-589/590 in the reaction. Ethacrynic acid was inhibitory in all conditions as expected (Fig. 3).

Sulfhydryl Content of Hsp90 and Hsp90 Peptides

As a next step in our investigations we were interested to know how many of the six cysteine side chains are accessible in native Hsp90. The amount of sulfhydryls accessible to the Ellman reagent in native Hsp90 is between three and four residues. After guanidinium–HCl treatment all the 6 sulfhydryl groups became exposed (Fig. 4). Judged by the same method, the sulfhydryls of peptide I and IIa were 70 to 80% reduced in our assay conditions (Fig. 4). Peptide concentrations were adjusted according to their sulfhydryl content throughout this study.

Protein Disulfide Oxidoreductase Activity of Hsp90 and Hsp90 Peptides

Encouraged by the presence of reductive cysteine residue(s) in Hsp90, we wanted to test, whether these residues are able to promote the reduction of disulfide bonds. A widely used method to monitor the reduction of disulfides is the reduction of insulin. In the generally accepted method of utilizing this process as a measure of protein disulfide oxidoreductase activity, the reducing agent, dithiothreitol, is added at suboptimal concentrations (20). In this way the presence of a protein disulfide oxidoreductase promotes the "recyclization" of dithiothreitol, achieving a faster separation of the A and B chains of insulin, and inducing the consequential aggregation of the B chain. In our initial experiments we found that the latency time and consequent insulin aggregation can be monitored best at pH 6.5, using 2 mM dithiothreitol. Analyzing the efficiency of Hsp90 to promote disulfide reduction it showed a negligible enhancement of insulin aggregation. In some experiments a small initial enhancement of the aggregation could be observed (marked with the arrow in Fig. 5). In contrast, DsbA, a bacterial protein disulfide isomerase (2) provoked a rapid aggregation of insulin B chains (Fig. 5). Larger amounts of Hsp90 induced an inhibition of insulin aggregation in accordance with the chaperone activity of the Hsp90 at greater or equimolar concentrations with insulin (31). To assess the disulfide reducing activity of Hsp90 peptides we have selected peptides I and IIa, since insulin has two negative charges at neutral pH, enabling it to interact with the neutral peptide I and the positive peptide IIa. However, neither peptide I, nor peptide IIa induced an enhancement of insulin aggregation (Fig. 5). Since the detection of insulin aggregation by light scattering requires relatively large amounts of insulin, and the aggregation is only an indirect measure of the reduction of insulin disulfide bonds, we also analyzed the reduction of insulin using FITC-labeled insulin (16, 17). Utilizing this assay, we were able to detect an

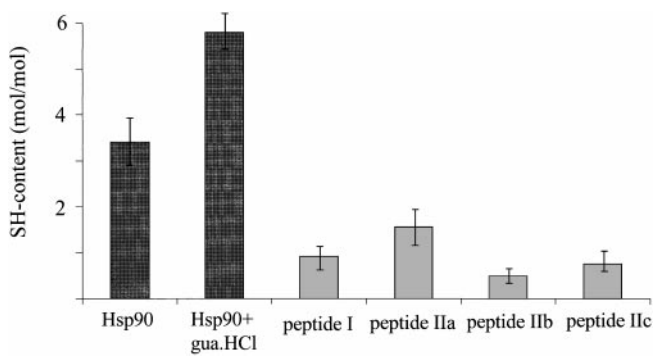

FIG. 4. Sulfhydryl content of Hsp90 and Hsp90 peptides. The amount of free sulfhydryl groups of Hsp90 was measured by the Ellman method (21) as described under Materials and Methods. 0.12 nmol of Hsp90 in the presence and absence of 6 M guanidinium–HCl, as well as 6 nmol of peptides I or IIa, respectively, were allowed to react with the Ellman reagent, and their sulfhydryl content was calculated using an extinction coefficient of 14,150 (22). Data are means ± SD of three independent experiments.

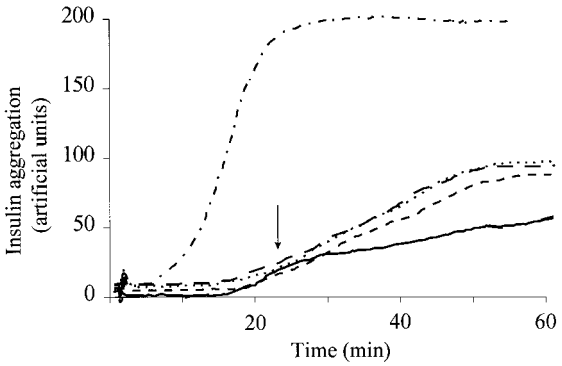

FIG. 5. Protein disulfide oxidoreductase activity of Hsp90 and Hsp90 peptides. Enhancement of insulin aggregation was detected as described under Materials and Methods. Aggregation of 50 μM insulin was monitored in the presence of 1, 10, or 10 μM Hsp90 (solid line), peptide I (long dashes), and peptide IIa (dotted line), respectively. Aggregation of insulin alone (dashed line) and insulin in the presence of 0.5 μM DsbA (alternating dots and dashes) as a positive control was also recorded. Curves are representatives of five independent experiments.

enhancement in the fluorescence of FITC-labeled insulin after the addition of thioredoxin as a positive control. At the initial phase of the reaction with thioredoxin a slight decrease in the fluorescence was also observed, which was due to the separation of insulin chains and the exposure of insulin-bound fluorescein to the aqueous environment. However, neither Hsp90 nor peptides I and IIa–IIc induced a significant fluorescence change in this assay (data not shown).

NADPH:Quinone Oxidoreductase Activity

Encouraged by the finding of Rao *et al.* (13) that ζ-crystallin possesses a NADPH:quinone oxidoreductase activity, we have tested whether purified Hsp90 has a similar activity. In contrast to postmitochondrial supernatants of rat liver homogenates used as a positive control, we failed to detect a NADPH:quinone oxidoreductase activity of purified rat liver Hsp90 using 1,2-naphthoquinone as a substrate. No further oxidation of NADPH occurred when the reaction was followed for 30 min. The addition of Ca^{2+} or Mg^{2+} ions did not change the negligible reaction rate with Hsp90. Hsp90 was also inactive, when tested with NADH as a reducing equivalent or with 9,10-phenanthrenequinone or juglone (5-hydroxy-1,4-naphthoquinone) as substrates. Similarly, when Hsp90 was mixed with hydroquinone and $NADP^+$ or NAD^+, there was no reduction of the nucleotides (data not shown).

Effect of the Redox Environment on Hsp90 Chaperone Activity

To assess the involvement of reactive cysteines in Hsp90 action further, we tested whether oxidized or reduced state of these cysteines affects the chaperone function of Hsp90. The dimeric mitochondrial enzyme citrate synthase is a well known, classical substrate for testing chaperone activity (23). Reactivation of chemically denatured citrate synthase was followed in the presence of Hsp90. After 60 min of renaturation citrate synthase regained approximately 30% of its initial enzyme activity. There was only a negligible reactivation in the absence of Hsp90. If we shifted the redox status of the renaturation buffer toward more oxidizing conditions by adding 1 mM dehydroascorbic acid, or 2.5 mM GSSG, the efficiency of the reactivation by Hsp90 strongly decreased: only around 15% of the initial enzyme activity was detectable after 60 min of renaturation, which is half of the efficiency of unoxidized Hsp90. The presence of 1 mM ascorbic acid in the renaturation buffer did not influence significantly the reactivation kinetics (Fig. 6). Neither dehydroascorbic acid, nor GSSG inhibited the citrate synthase activity by themselves in the concentrations mentioned above (data not shown).

DISCUSSION

As one of the major findings of the present report we have demonstrated that Hsp90 is able to reduce cytochrome c. The *N*-ethylmaleimide sensitivity suggests that the effect is mediated by sulfhydryl groups of Hsp90. According to the Ellman determination there are three to four accessible sulfhydryl groups per native Hsp90 monomer. The efficient inhibition of Hsp90-

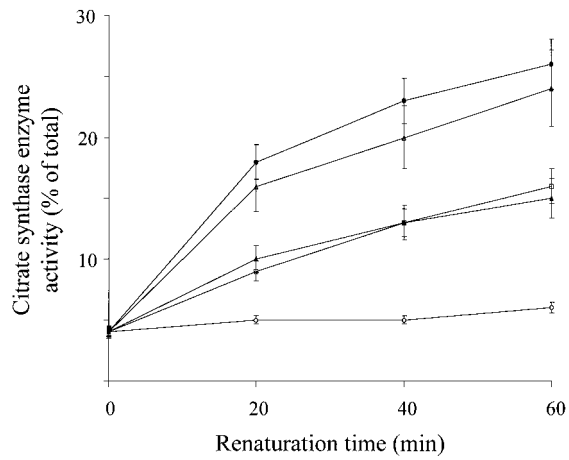

FIG. 6. Effect of redox environment on the chaperone activity of Hsp90. Chaperone activity of Hsp90 was monitored using the test system citrate synthase (23) as described under Materials and Methods. Chemically denatured citrate synthase was reactivated in the presence of Hsp90 (filled circles). The redox status of Hsp90 was changed by adding 2.5 mM GSSG (open squares), 1 mM dehydroascorbic acid (filled triangles), or 1 mM ascorbic acid (open triangles) to the renaturation buffer. No reactivation was detected in the absence of Hsp90 (open circles). Curves are representatives of three independent experiments.

TABLE III

Standard Redox Potentials of Redox Systems and Protein Disulfide Isomerases Used in This Study

Name	Redox potential (V)	Reference
Redox system		
Cytochrome c(Fe^{3+})/cytochrome c(Fe^{2+})	+0.25	52
Insulin(A-S-S-B)/insulin(A-SH) + insulin(B-SH)	−0.20[a]	53
Dithiothreitol(S-S)/ dithiothreitol($-SH)_2$	−0.32	54
$NADP^+$/NADPH	−0.32	52
Protein disulfide isomerase		
DsbA(S-S)/DsbA($-SH)_2$	−0.09	2
Thioredoxin(S-S)/ thioredoxin($-SH)_2$	−0.27	2, 52

[a] The apparent standard redox potential of the two disulfides of insulin was calculated from its redox equilibrium with thioredoxin.

induced cytochrome c reduction by the vicinal thiol-specific SH reagents, arsenite and $CdCl_2$ (30), indicates the involvement of the single vicinal thiol pair of Hsp90, the highly conserved Cys-589/590 pair in the reaction. Indeed, peptide IIa containing these thiols (see sequence in Table II) was able to reduce cytochrome c. On the other hand, a smaller contribution of Cys521 (contained by peptide I) in Hsp90-mediated cytochrome c reduction cannot be excluded, since peptide I was a better reductive agent for cytochrome c than peptide IIa and its efficiency reached that of Hsp90 itself.

Contrary to its cytochrome c reducing activity, Hsp90 proved to be rather ineffective as a reducing agent for the disulfide bonds of insulin, or in other words, Hsp90 does not possess significant protein disulfide oxidoreductase activity. This finding is in good agreement with the negligible protein disulfide oxidoreductase activity of Grp94, the highly homologous counterpart of Hsp90 in the lumen of the endoplasmic reticulum (33). What can be the reason of this marked activity difference of Hsp90 in the two redox systems? If we compare the standard redox potential of the cytochrome c and insulin redox systems (Table III), it is readily apparent that cytochrome c can be reduced much easier than insulin. Thus the redox potential of Hsp90 might be higher than that of the "normal" protein disulfide isomerases (exemplified in Table III by the bacterial protein disulfide isomerase, DsbA). This ability of the protein to act as a reducing agent of cytochrome c, however, prevents Hsp90 from acting as a protein disulfide oxidoreductase at the −0.23-V estimated redox potential (34) of the cytosol. Besides these differences steric factors might also contribute to the different behavior of Hsp90 toward cytochrome c and insulin. Reduced sulfhydryl groups of Cys-481, Cys-656, and Cys-661 of the glucocorticoid receptor are necessary for the hormone binding and translocation of the receptor. Though Hsp90 plays an important role in the maturation of the receptor, in light of the above findings it seems unlikely that Hsp90 participates in the protection of these sulfhydryl groups, which are reduced by thioredoxin in an NADPH-dependent manner (35, 36).

The other interesting finding of the present report that oxidizing buffer conditions impair the chaperone activity of Hsp90 toward its usual test substrate, citrate synthase. This provides yet another example for the regulation of Hsp90 function by the redox status of this chaperone.

Reactive cysteines are involved in the interaction of Hsp90 with the heme-regulated eIF-2α kinase (8) and molybdate, a stabilizer of Hsp90-target interactions (9). Cysteines also play an important role in the function of other molecular chaperones, such as Hsp33 (14), Hsp70 (29), or α-crystallin (37). Interestingly, reactive cysteines are usually found in the vicinity of chaperone nucleotide binding sites such as the ATP-binding site of Hsp70 (29). The most probable Hsp90 cysteines, the Cys-589/590 pair is in the neighborhood of the second, putative low-affinity nucleotide binding site of Hsp90 (7, 27, 38). However, ATP did not modulate the Hsp90-induced reduction of cytochrome c, which indicates no direct competition between ATP and cytochrome c binding to Hsp90.

Cytochrome c-derived peptides are excellent substrates of the 70-kDa molecular chaperones (39, 40) and, quite surprisingly, cytochrome c itself may behave as a molecular chaperone, promoting the renaturation of ribonuclease (41, 42). Cytochrome c also stimulates α-synuclein aggregation in Lewy body disease (43). These reports may indicate possible interactions of "conventional chaperones" with cytochrome c and their cooperation in chaperone action. However, in our initial experiments we did not find any functional cooperation between isolated Hsp90 and cytochrome c in refolding of firefly luciferase (data not shown), which suggests that the chaperone cooperation between Hsp90 and cytochrome c, if it exists, may appear at a higher level of organization, at the foldosome.

Under normal conditions cytochrome c is a peripheral membrane protein of mitochondria. What are the chances of an *in vivo* interaction between cytochrome c and "conventional," cytoplasmic molecular chaperones, such as Hsp90? Cytochrome c is released from the outer surface of the inner mitochondrial membrane to the cytosol at the early steps of apoptosis and can trigger the activation of the apoptosis-specific DEVD-specific proteases, called caspases by binding to the complex of apoptosis-activating factor 1 and dATP (44–47). On the other hand, apoptosis and the stress re-

sponse are highly interrelated and altered expression of heat shock proteins exerts a great influence on the development of apoptosis (48). Recently thusfar unidentified cytoplasmic proteins which had been activated by caspase 8 were reported to be involved in triggering the apoptotic release of cytochrome c (49–51). Hsp90 harbors two caspase cleavage sequences, DEDE (amino acids 691–694 (25)) for type II caspases and LEGD (amino acids 711–714 (25)) for type III caspases in its extreme C-terminus. Indeed, Hsp90 can be cleaved by caspases *in vitro* (Cs. Sőti, T. Schnaider, P. Bauer, and P. Csermely, unpublished observations). These findings raise the possibility that redox reaction-related interactions of cytochrome c and cytoplasmic chaperones may play a role in the development or regulation of apoptotic events. Unfortunately dATP did not influence the reduction of cytochrome c by Hsp90 (data not shown), and our initial experiments did not show any significant association of Hsp90 to cytochrome c affinity columns (P. Bauer and P. Csermely, unpublished observations), which shows the necessity of more complicated experiments to decide if Hsp90 is involved in the regulation of the cytochrome c-mediated apoptotic pathway.

ACKNOWLEDGMENTS

This work was supported by research grants from ICGEB, Hungarian Science Foundation (OTKA-T25206, T30841), Hungarian Ministry of Social Welfare (ETT-21/00), and the Volkswagen Foundation (I/73612). P.C. is an International Research Scholar of the Howard Hughes Medical Institute. We thank Drs. Tamás Schnaider and Csaba Sőti (Department of Medical Chemistry, Semmelweis University, Budapest, Hungary) for their advice and help in the purification of Hsp90, Dr. Michael Oke Sunday (Department of Medical Chemistry, Semmelweis University, Budapest, Hungary) for his participation at the initiation of these studies, Drs. Alejandro P. Heuck and Ricardo A. Wolosiuk (Instituto de Investigaciones Bioquimicas, Universidad de Buenos Aires, Buenos Aires, Argentina) for their kind gift of the FITC-labeled insulin, and Ms. Katalin Mihály (Department of Medical Chemistry, Semmelweis University, Budapest, Hungary) for her excellent technical support.

Note added in proof. After our manuscript was accepted we became aware of the recent paper by Pandey *et al.* (55) which states that Hsp90 inhibits the cytochrome c-mediated initiation of the apoptotic cascade. Though, in agreement with our initial experiments, Pandey *et al.* found that Hsp90 does not stably associate with cytochrome c, the two proteins are interacting with each other as parts of a larger cytoplasmic complex. These findings strengthen our suggestion for the participation of Hsp90 redox activity in the regulation of the apoptotic machinery.

REFERENCES

1. Rietsch, A., and Beckwith, J. (1998) *Annu. Rev. Genet.* **32,** 163–184.
2. Raina, S., and Missiakas, D. (1997) *Annu. Rev. Microbiol.* **51,** 179–202.
3. Bukau, B., and Horwich, A. L. (1998) *Cell* **92,** 351–366.
4. Csermely, P. (1997) *Trends Biochem. Sci.* **22,** 147–149.
5. Buchner, J. (1999) *Trends Biochem Sci.* **24,** 136–141.
6. Pratt, W. B., and Toft, D. O. (1997) *Endocr. Rev.* **18,** 306–360.
7. Csermely, P., Schnaider, T., Sőti, Cs., Prohászka, Z., and Nardai, G. (1998) *Pharmacol. Ther.* **79,** 129–168.
8. Mendez, R., Moreno, A., and de Haro, C. (1992) *J. Biol. Chem.* **267,** 11500–11507.
9. Sőti, Cs., Radics, L., Ichiro, Y., and Csermely, P. (1998) *Eur. J. Biochem.* **255,** 611–617.
10. Hartson, S. D., Thulasiraman, V., Huang, W., Whitesell, L., and Matts, R. L. (1999) *Biochemistry* **38,** 3837–3849.
11. Simpkins, C. O., Fogarty, K. W., II, and Nhamburo, P. (1993) *Life Sci.* **52,** 1487–1492.
12. De Crouy-Chanel, A., Kohiyama, M., and Richarme, G. (1995) *J. Biol. Chem.* **270,** 22669–22672.
13. Rao, P. V., Krishna, C. M., and Zigler, J. S., Jr. (1992) *J. Biol. Chem.* **267,** 96–102.
14. Jakob, U., Muse, W., Eser, M., and Bardwell, J. C. A. (1999) *Cell* **96,** 341–352.
15. Bódi, J., Süli-Vargha, H., Ludányi, K., Vékey, K., and Orosz, G. (1997) *Tetrahedron Lett.* **38,** 3293–3296.
16. Heuck, A. P., and Wolosiuk, R. A. (1997) *J. Biochem. Biophys. Methods* **34,** 213–225.
17. Heuck, A. P., and Wolosiuk, R. A. (1997) *Anal. Biochem.* **248,** 94–101.
18. Csermely, P., and Kahn, C. R. (1991) *J. Biol. Chem.* **266,** 4943–4950.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
20. Holmgren, A. (1979) *J. Biol. Chem.* **254,** 9627–9632.
21. Ellman, G. L., Courtney, D., Andres, V., Jr., and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7,** 88–95.
22. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1979) *Anal. Biochem.* **94,** 75–81.
23. Jacob, U., Gaestel, M., Engel, K., and Buchner, J. (1993) *J. Biol. Chem.* **268,** 1517–1520.
24. Fiser, A., Cserző, M., Tüdős, É., and Simon, I. (1992) *FEBS Lett.* **302,** 117–120.
25. Poellinger, L., Wikström, A.-C., Gustafsson, J. A. (1992) *J. Steroids Biochem. Mol. Biol.* **42,** 813–822.
26. Frömmel, C., and Preissner, R. (1990) *FEBS Lett.* **277,** 159–163.
27. Callebaut, I., Catelli, M. G., Portetelle, D., Meng, X., Cadepond, F., Burny, A., Baulieu, E.-E., and Mornon, J.-P. (1994) *C. R. Acad. Sci. Paris* **317,** 721–729.
28. Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) *Cell* **90,** 65–75.
29. Liu, Q., Levy, E. J., and Chirico, W. J. (1996) *J. Biol. Chem.* **271,** 29937–29944.
30. Gunther, T., and Ahlers, J. (1976) *Arzneimittelforschung* **26,** 13–14.
31. Simons, S. S., Chakraborti, P. K., and Cavanaugh, A. H. (1990) *J. Biol. Chem.* **265,** 1938–1945.
32. Scheibel, T., Weikl, T., and Buchner, J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 1495–1499.
33. Rupp, K., Birnbach, U., Lundström, J., Nguyen Van, P., and Söling, H.-D. (1994) *J. Biol. Chem.* **269,** 2501–2507.
34. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* **257,** 1496–1502.
35. Simons, S. S., Jr., and Pratt, W. B. (1995) *Methods Enzymol.* **251,** 406–422.
36. Okamoto, K., Tanaka, H., Ogawa, H., Makino, Y., Eguchi, H., Hayashi, S., Yoshikawa, N., Poellinger, L., Umesono, K., and Makino, I. (1999) *J. Biol. Chem.* **274,** 10363–10371.

37. Cherian, M., Smith, J. B., Jiang, X.-Y., and Abraham, E. C. (1997) *J. Biol. Chem.* **272,** 29099–29103.
38. Sőti, Cs., and Csermely, P. (1998) *J. Biosci.* **23,** 347–352.
39. Greene, L. E., Zinner, R., Naficy, S., and Eisenberg, E. (1995) *J. Biol. Chem.* **270,** 2967–2973.
40. Vanbuskirk, A., Crump, B. L., Margoliash, E., and Pierce, S. K. (1989) *J. Exp. Med.* **170,** 1799–1809.
41. Ramakrishna Kurup, C. K., Raman, T. S., Jayaraman, J., and Ramasarma, T. (1964) *Biochim. Biophys. Acta* **93,** 681–683.
42. Ramakrishna Kurup, C. K., Raman, T. S., and Ramasarma, T. (1964) *Biochim. Biophys. Acta* **113,** 255–276.
43. Hashimoto, M., Takeda, A., Hsu, L. J., Takenouchi, T., and Masliah, E. (1999) *J. Biol. Chem.* **274,** 28849–28852.
44. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) *Science* **275,** 1129–1132.
45. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275,** 1132–1136.
46. Green, D. R., and Reed, J. C. (1998) *Science* **281,** 1309–1312.
47. Wang, H.-G., Rapp, U. R., and Reed, J. C. (1996) *Cell* **87,** 629–638.
48. Punyiczki, M., and Fésüs, L. (1998) *Ann. NY Acad. Sci.* **851,** 67–74.
49. Han, Z. Y., Li, G., Bremner, T. A., Lange, T. S., Zhang, G. H., Jemmerson, R., Wyche, J. H., and Hendrickson, E. A. (1998) *Cell Death Differ.* **5,** 469–479.
50. Steemans, M., Goossens, V., VandeCraen, M., Vanherreweghe, F., Vancompernolle, K., DeVos, K., Vandenabeele, P., and Grooten, J. (1998) *J. Exp. Med.* **188,** 2193–2198.
51. BossyWetzel, E., and Green, D. R. (1999) *J. Biol. Chem.* **274,** 17484–17490.
52. Rawn, J. D. (1989) "Biochemistry," Neil Patterson, Burlington, NC.
53. Krause, G., Lundström, J., Barea, J. L., de la Cuesta, C. P., and Holmgren, A. (1991) *J. Biol. Chem.* **266,** 9494–9500.
54. Hawkins, H. C., de Nardi, M., and Freedman, R. B. (1991) *Biochem. J.* **275,** 341–348.
55. Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. M., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E. S., Kufe, D., and Kharbanda, S. (2000) *EMBO J.* **19,** 4310–4322.