Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1751–1755, March 1994
Cell Biology

Heat stress induces hsc70/nuclear topoisomerase I complex formation *in vivo*: Evidence for hsc70-mediated, ATP-independent reactivation *in vitro*

(thermotolerance/protein folding/T cells)

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Communicated by Mary Lou Pardue, November 5, 1993

ABSTRACT **We previously demonstrated that in murine T cells thermotolerance correlated with heat shock protein 70 (hsp70) synthesis and protection of nuclear type I topoisomerase (topo I). Topo I activity returned to normal levels following heat stress even in cells not rendered thermotolerant by a prior heat shock. Recovery of topo I activity was not dependent on *de novo* protein synthesis, suggesting that the cell possesses a pathway(s) for refolding this nuclear protein. In this report we demonstrate that topo I and hsc70, the constitutively produced member of the hsp70 family, associated *in vivo* during heat stress. That this association may play a physiologically important role in protecting topo I activity from heat stress was suggested by the observation that hsc70 protected topo I from heat inactivation *in vitro*. hsc70 but not actin also reactivated previously heat-denatured topo I in a dose-dependent fashion. However, refolding of heat-denatured topo I by purified hsc70 was inefficient relative to a hsc70-containing cell lysate. Protection from heat inactivation as well as reactivation by hsc70 did not require exogenous ATP. Similarly, reactivation by the cell lysate was not inhibited by ADP or a nonhydrolyzable analogue of ATP. Thus, our studies suggest that nuclear topo I complexes with hsc70 during heat stress, which may explain, at least in part, why hsp70 proteins accumulate in the nucleus, particularly the nucleolus. This interaction may limit heat-induced protein damage and/or accelerate restoration of protein function in an ATP-independent reaction.**

Elevated temperatures induce a transient resistance to subsequent heat exposure in cells from various organisms. This phenomenon, termed acquired thermotolerance, is associated with the rapid and preferential synthesis of a small set of highly conserved proteins, the heat shock proteins (hsps; reviewed in ref. 1). Some hsps, such as the 70-kDa cognate hsp (hsc70), are constitutively produced in nonstressed cells and their synthesis is only moderately enhanced following heat shock. In contrast, hsp70, a distinct but closely related protein to hsc70, is not constitutively produced in rodent cells but can rapidly become an abundantly synthesized protein following heat stress. A number of studies suggest that hsps, particularly members of the hsp70 family, play a role in the acquisition of thermotolerance; however, this evidence is largely circumstantial and rests on a correlation of cellular levels of hsp70 with thermotolerance (2–4). During heat stress hsc70 and hsp70 both leave the cytoplasm and nucleoplasm and rapidly accumulate in large amounts in the nucleolus (5). This migration is transient because removal of the stress results in redistribution back to the nucleoplasm and cytoplasm. Understanding why members of the hsp70 family accumulate in the nucleolus during heat stress will undoubtedly provide insights on hsp70 function and its role in thermotolerance.

Previous studies from our laboratory demonstrated that heat stress initially inhibited DNA replication in thermotolerant and control cells. However, following an incubation at a nonstress temperature (37°C), thermotolerant cells recovered their ability to replicate DNA at an accelerated rate relative to the control population. This recovery was correlated with the accumulation of elevated levels of hsc70 and hsp70 in thermotolerant cells (6). In these studies we suggested that hsp70 proteins may protect a critical enzyme(s) required for DNA synthesis from heat-induced damage. Because nuclear type I topoisomerase (topo I) is an important enzyme in DNA replication and transcription (reviewed in ref. 7) and is concentrated in the nucleolus (8), we subsequently assessed the relationship between DNA replication and topo I activity following heat stress in control and thermotolerant cells. These studies demonstrated a good correlation between cellular levels of hsp70 proteins, topo I activity, and the ability to reinitiate DNA replication (9). Further studies revealed that heat stress, followed by a rest period at 37°C, resulted in the reappearance of normal levels of topo I activity in thermotolerant and control cells, even in the absence of *de novo* protein synthesis (9). The role of hsp70 proteins, if any, in limiting heat-induced inactivation of topo I as well as its reactivation following heat denaturation was not evaluated in these earlier studies. In this report, we present evidence that hsc70 associates with topo I in cells subjected to heat stress. Furthermore, at least *in vitro*, purified hsc70 can protect topo I from heat inactivation *in vitro* as well as reactivate heat-denatured topo I. These properties closely mirror the events that occur in cells following heat stress and suggest a role for hsc70 in protecting and regenerating the catalytic activity of this enzyme *in vivo*.

MATERIALS AND METHODS

Induction and Evaluation of Thermotolerance. The isolation of purified T cells as well as the induction and evaluation of thermotolerance has been described (6, 10). Data expressed as % of control were calculated from the following formula: (units of topoisomerase activity after heat stress/units of topoisomerase activity before heat stress) × 100.

Antibodies, Proteins, and Preparation of Cell Lysates. Monoclonal antibodies (mAbs) against hsp70 were from commercial sources (Oncogene Science or StressGen Biotechnologies, Victoria, BC Canada). Purified actin, ATP, apyrase, and adenosine 5′-[β,γ-imido]triphosphate (AMP-

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Abbreviations: mAb, monoclonal antibody; topo I, type I topoisomerase; hsp, heat shock protein; AMP-*P*[NH]*P*, adenosine 5′-[β,γ-imido]triphosphate; RNAP, RNA polymerase.

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P[NH]*P*), a nonhydrolyzable analogue of ATP, were obtained from Sigma; purified hsc70 was obtained from Stress-Gen Biotechnologies. T-cell lysates were prepared in phosphate-buffered saline containing 0.1% Triton X-100, 5 mM $MgCl_2$, and a cocktail of protease inhibitors. Following a 20-min incubation, the lysate was centrifuged (5 min, 14,000 × *g*) and the supernatant was aliquoted and stored at −70°C. All steps were performed at 4°C. Protein concentration was determined using a bicinchoninic acid protein assay reagent (Pierce) following the manufacturer's recommendations. Topo I activity was present in the cell lysates and was therefore removed by phosphocellulose adsorption chromatography. Residual topo I activity, if still present in the lysate, was subtracted from the total units of topo I obtained following reactivation of heat-denatured topo I by the cell lysate.

Topo I Assay. Topo I assays were performed essentially as described in Lazarus *et al.* (11). Briefly, highly supercoiled plasmid pUC19 was treated with topo I for 30 min at 30°C in assay cocktail. The removal of supercoils was assessed by the production of a ladder of slow-moving bands on an agarose gel. The DNA was visualized after staining and photographed, and the amount of DNA in each band was quantified by densitometry. One unit of activity is the amount of enzyme necessary to remove one-half of the supercoils from 300 ng of input DNA.

Heat Denaturation and Reactivation Assay. Purified topo I (1 μl) was mixed with 1 μl of either assay cocktail (11), actin, or hsc70. Samples were then diluted with 8 μl of assay cocktail and preincubated for 5 min at 30°C prior to transfer to 45°C. At the indicated time points at 45°C, samples were transferred to 30°C and residual topo I activity was measured as described above.

To assess the ability of specific proteins or a cell lysate to reactivate heat-denatured topo I, purified topo I was incubated at 45°C for 10 min in assay cocktail. Aliquots (1 μl) of heat-inactivated topo I were added to 8 μl of assay cocktail and various concentrations of either the indicated protein (1 μl) or cell lysate. Following a 30-min incubation at 30°C to allow for reactivation of the enzyme, pUC19 DNA (1 μl) was added and topo I activity was assayed. The values presented in Figs. 2 and 3 represent the mean of triplicate determinations and are representative of at least three independent experiments.

Immunoprecipitation and Immunoblot Analysis. T cells were given a modest heat shock as described (9), rested for 12 hr at 37°C to allow for synthesis of hsp70, and then heat shocked at 45°C for 30 min. Lysates were then immediately prepared in the presence of ATP (2.5 mM) or apyrase (10 units/ml). Following a 30-min incubation at 4°C, hsp70 proteins were immunoprecipitated from the lysate under nondenaturing conditions (12) using mAbs that were either specific for hsp70 (W27) or recognized hsc70 and hsp70 (N27F3-4). Immunoprecipitates were analyzed for the presence of hsp70 and/or hsc70 by immunoblot analysis as described (10). Immunoprecipitates obtained using mAb to hsp70 were also analyzed for copurified topo I by immunoblot analysis using a rabbit antiserum specific for topo I (kindly provided by L. Liu, University of Medicine and Dentistry, New Jersey–R. W. Johnson Medical School, NJ). Rabbit anti-topo I antibody was detected with a biotinylated goat anti-rabbit IgG (Oncogene Science) followed by an alkaline phosphatase-conjugated streptavidin reagent (Oncogene Science) and the appropriate substrate (Vector Laboratories).

Isolation of Topoisomerase. DNA topo I was prepared from calf thymus nuclei by the procedure of Schmitt *et al.* (13) with modifications (14). Approximately 10 mg of purified enzyme was recovered from 1 kg of fresh thymus.

RESULTS

As noted previously, topo I activity was inactivated by heat stress more severely in control than thermotolerant cells. However, control cells rested at 37°C recovered their topo I activity with time even in the absence of *de novo* protein synthesis (9). This suggests that the cell possesses a pathway(s) for refolding denatured proteins in the nucleus. Because heat stress induces the migration of hsp70 proteins into the nucleolus, a site where topo I is heavily concentrated, we tested the possibility that members of the hsp70 family bind to heat-denatured topo I and as a result of this interaction minimize thermal damage and/or accelerate recovery of the catalytic activity of this enzyme. If this view is correct, some topo I/hsp70 complexes should exist in the cell following thermal injury. To assess this possibility, thermotolerant cells were heat shocked and lysed, and the cell lysates were immediately treated with apyrase to deplete ATP. In the absence of ATP, hsp70 proteins should remain bound to their substrates following heat stress and therefore copurify with immunoprecipitated hsp70 proteins. It can be seen from Fig. 1*A* that mAb W27 immunoprecipitated hsp70, whereas mAb N27F3-4 (N27) immunoprecipitated approximately the same quantities of hsc70 and hsp70. When these same immunoprecipitates were analyzed for the presence of topo I, we detected topo I in the N27 but not W27 immunoprecipitates (Fig. 1*B*). The rabbit anti-topoisomerase serum used in this blot detects the whole enzyme (≈100 kDa) as well as proteolytic fragments, particularly a major peptide at ≈70 kDa. This major 70-kDa fragment was copurified using mAb N27 but was absent from the purified topo I preparation. The observation that immunoprecipitation with mAb W27 did not copurify topo I indicates that nonspecific protein–protein interactions were not responsible for the presence of topo I in the N27 immunoprecipitate. It should be noted that the topo I blot was incubated with the substrate for several hours, which accounts for the very strong topo I signal. This was done in an effort to detect small quantities of topo I that may have copurified with mAb W27. Additional studies demonstrated that heat stress was required to induce hsp70/topo I complex formation—that is, immunoprecipitation of hsp70 proteins from control lysates (non-heat shocked) did not copurify topo I. Indeed, few radiolabeled proteins were immunoprecipitated from control lysates; whereas, a large

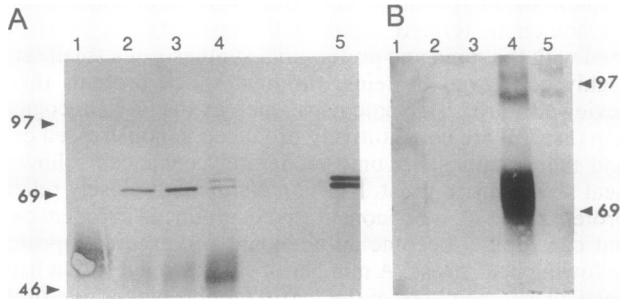

FIG. 1. hsc70 and nuclear topo I associate *in vivo* following thermal injury. Western blot analysis was performed on immunoprecipitated proteins from heat-shocked lysates. The blot was split with one section probed for hsp70 proteins (*A*) and the remaining blot probed for topo I (*B*). (*A*) Immunoprecipitates prepared using the following mAbs: lane 1, IgG2a against an irrelevant antigen (negative control); lanes 2 and 3, W27; lane 4, N27; lane 5, lysate from cells given multiple heat shocks (positive control). Immunoprecipitates were prepared in the presence of either apyrase (lanes 1, 3, and 4) or 2.5 mM ATP (lane 2). (*B*) Lanes 1–4 are the same samples as in lanes 1–4 in *A*. Lane 5 contains 5 ng of purified topo I. The numbers on the sides of the blots represent the locations of molecular mass markers expressed in kDa. The bands (*A*) that are present between the 69- and 46-kDa markers are murine immunoglobulin heavy chains.

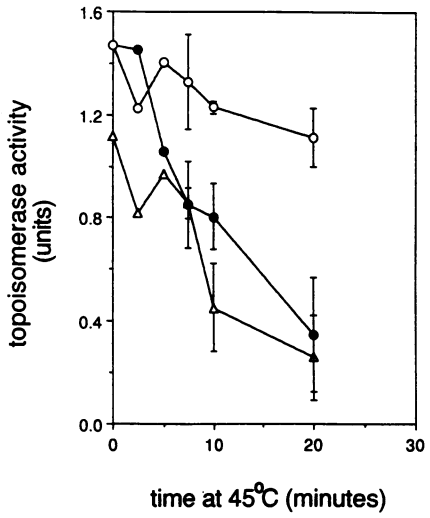

FIG. 2. hsc70 protects topo I from thermal inactivation *in vitro*. Purified topo I (3.8 ng) was incubated at 45°C in the presence of assay cocktail (△), actin (16 ng, ●), or hsc70 (26.6 ng, ○). These concentrations represented a protein:topoisomerase molar ratio of 30:1. At the indicated times, samples were removed and tested for residual topo I activity. No ATP was added to the reaction mixtures during either heat inactivation or enzyme analysis. Values presented represent the means of triplicate determinations ± SD. Error bars for some values have been omitted for clarity.

number of radiolabeled proteins were present in the hsp70 immunoprecipitates obtained from heat-shocked cells (data not shown). Thus, following heat stress, members of the hsp70 family migrate into the nucleolus where hsc70 and perhaps hsp70 physically associate with topo I. We speculate that this physical interaction contributes to stabilizing the catalytic site during heat inactivation, although how this is achieved mechanistically remains to be clarified. The failure to copurify topo I with W27 may indicate that only hsc70 associates with topoisomerase *in vivo* or, alternatively, W27 binding to hsp70 dissociates topo I from hsp70. Further studies will be required to assess each of these possibilities.

In rodent cells hsc70 is the only member of the hsp70 family that is synthesized at detectable levels under non-stress conditions. Thus, during an initial heat shock, hsc70 and not hsp70 would function to minimize or limit the extent of cellular injury. In view of these kinetics and the results presented in Fig. 1, we sought more direct evidence for a role of hsc70 in protecting topo I from heat stress. For this reason, we monitored the rate of heat inactivation of topo I either alone or in the presence of hsc70. In the absence of any other protein, purified topo I quickly lost most of its catalytic activity at 45°C (Fig. 2). The addition of purified actin did little to change the inactivation kinetics. However, the presence of the same molar quantity of purified hsc70 (≈13 hsc70 monomers per enzyme) markedly reduced the rate of thermal inactivation of this enzyme, with >4-fold more topo I activity in the presence of hsc70. This protection was achieved in the absence of any exogenous ATP.

In vivo, topo I activity recovers following heat stress in control cells that initially contain hsc70 and undetectable levels of inducible hsp70 (9). It was of interest therefore to determine if we could duplicate this activity *in vitro* using a cell lysate prepared from control cells. For these experiments, purified topo I was first heat inactivated and then mixed with various concentrations of cell lysate. The reaction mixture was then incubated to allow for reactivation of the enzyme and subsequently tested for topo I activity. The results of this experiment, presented in Fig. 3 *Upper*, demonstrated that there was a dose-dependent reactivation of topo I activity by the control cell lysate. That hsc70 contributed to the refolding activity present in the cell lysate was suggested by the observation that purified hsc70 also reac-

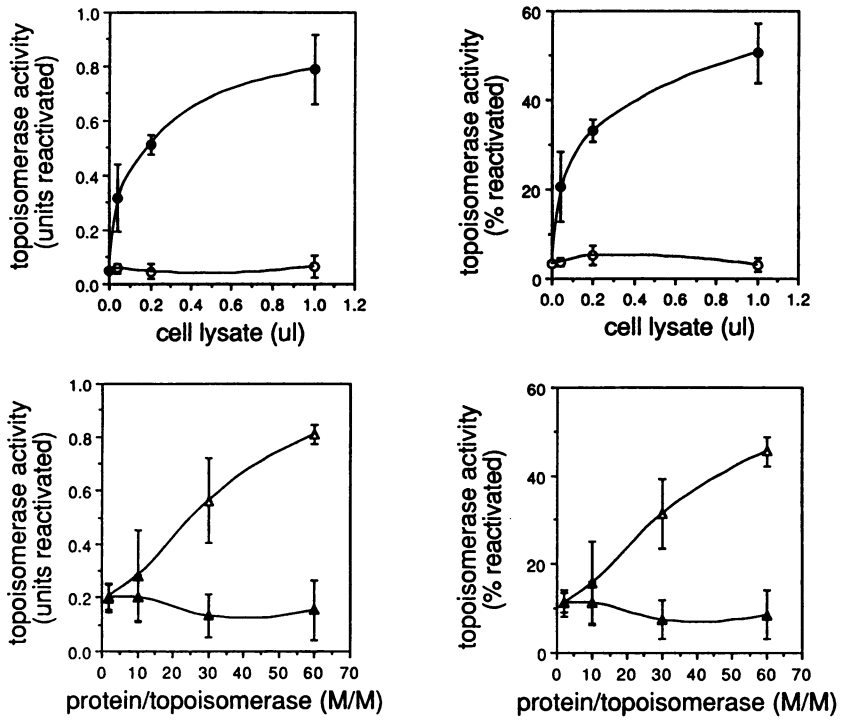

FIG. 3. Reactivation of heat-denatured topo I. (*Upper*) Purified topo I (3.8 ng) was heat-inactivated at 45°C for 10 min and then mixed with the indicated volume of either assay cocktail (○) or cell lysate (●) obtained from control (thermosensitive) T cells. All samples were brought to a final volume of 10 μl with assay cocktail and then incubated for 30 min at 30°C to allow for reactivation of the enzyme. Topo I activity was then evaluated. Protein concentration of the cell lysate was 106 ng/μl. (*Lower*) A similar protocol as that described above was followed with the exception that heat-inactivated topo I was incubated with various concentrations of either actin (▲) or hsc70 (△).

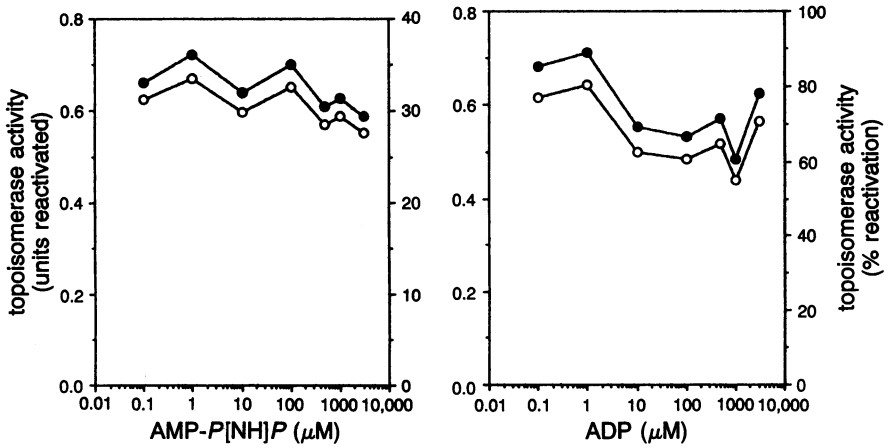

FIG. 4. Effect of AMP-*P*[NH]*P* and ADP on reactivation of heat-inactivated topo I. The protocol was similar to that described in the legend to Fig. 3 with the exception that the cell lysate was incubated for 15 min at 35°C with either AMP-*P*[NH]*P* (*Left*) or ADP (*Right*) prior to the addition of heat-inactivated topo I. Topo I not subjected to heat shock contained either 1.66 units (AMP-*P*[NH]*P* experiment) or 0.73 unit (ADP experiment). Heat shock and recovery at 30°C in the absence of the cell lysate resulted in almost no reactivation of enzyme activity in both experiments (<0.080 unit). Data are expressed as either units reactivated (○) or % reactivation (●).

tivated heat-denatured topo I in a dose-dependent fashion (Fig. 3 *Lower*). Higher ratios of hsc70:topoisomerase did not result in a greater yield of enzyme activity (data not shown). Refolding of topo I was not due to nonspecific protein–protein interactions because actin, at identical molar ratios, failed to reactivate topo I.

The ability of the cell lysate to efficiently refold heat-denatured topo I cannot be attributed solely to hsc70 present in the lysate. For example, the addition of 0.2 μl of lysate to the reaction mixture resulted in a concentration of hsc70 of $\approx 5.5 \times 10^{-11}$ M. This value was obtained by immunoblot analysis using a calibration curve generated with purified hsc70 (data not shown). Since the reaction mixture contained 1.14×10^{-8} M topoisomerase, there was only one hsc70 monomer per 208 topo I molecules. In contrast, an hsc70:topo I ratio of 60:1 was required to reactivate a similar percentage of the enzyme when the reaction mixture contained only purified hsc70 (Fig. 3). These results suggest that hsc70 becomes highly efficient ($\approx 10^4$ times) in the presence of other cytoplasmic factors. This view is consistent with the observation that for some proteins, regeneration of function requires the cooperation of several constitutively expressed cognates operating either together or in tandem (15–18). It is also possible that the cell possesses another refolding pathway that is independent of hsc70. To discriminate between these two possibilities, we have recently depleted a cytosol of hsc70 by passage through an ATP-affinity column and observed that the depleted cytosol was unable to reactivate heat-denatured topo I. Recovery of catalytic activity was achieved by the addition of proteins eluted from the ATP-affinity column or by purified hsc70 (unpublished data). In addition, admixture of hsc70 and a depleted cytosol at concentrations where neither had refolding activity resulted in a synergistic reactivation of heat-denatured topo I (unpublished data). Thus, these studies support the view that hsc70 is an obligatory participant in the refolding reaction responsible for regenerating native topo I *in vivo* and that hsc70 interacts with a component(s) in the cytoplasm that dramatically augments its refolding activity.

hsp70 has been shown to have two binding sites—an N-terminal domain that binds ATP and a C-terminal domain that binds peptides and unfolded or misfolded proteins (reviewed in ref. 19). *In vitro*, hsc70 utilizes the energy of ATP hydrolysis to release bound substrate proteins presumably in an active conformation (20). Although we have obtained reactivation of topo I by the cell lysate and purified hsc70 in the absence of exogenous ATP, it could be argued that sufficient ATP is bound to hsc70 for refolding to occur. We tested this possibility by incubating the lysate with various concentrations of AMP-*P*[NH]*P* prior to the addition of the substrate, heat-inactivated topo I. The results, presented in Fig. 4, demonstrate that ≈30% of the catalytic activity was reactivated by the cell lysate. However, the presence of even large concentrations of AMP-*P*[NH]*P* did not prevent refolding of the enzyme mediated by components in the cell lysate (Fig. 4 *Left*). This result cannot be explained by ATPase activity in the lysate because incubation of the lysate with AMP-*P*[NH]*P* did not result in hydrolysis of AMP-*P*[NH]*P* (data not shown). We also performed similar competition experiments with ADP, which has a higher binding affinity for hsp70 than ATP. ADP inhibits ATP-induced dissociation of the hsp70–protein complex and therefore prevents refolding of the substrate (21). We found that at all concentrations tested, ADP failed to inhibit reactivation of heat-denatured topo I (Fig. 4 *Right*). Thus our studies suggest that nuclear topo I can be refolded *in vivo* by a pathway that does not require ATP hydrolysis.

DISCUSSION

It is well documented that following thermal stress, hsp70 proteins migrate from the cytosol into the nucleus; however, in the nucleus preferential accumulation is observed within the nucleolus. This suggests that denatured proteins or structures within the nucleolus are targeted for protection by hsp70 proteins and that this protection is important in recovery from thermal injury. However, the nuclear substrate(s) that interacts with hsp70 has not been previously identified. In this study we demonstrate that immunoprecipitation of hsp70 from a heat-shocked extract copurified nuclear topo I. The predominant species in the immunoprecipitate was a 70-kDa fragment of topo I that may have been generated during thermal injury or as a result of proteolytic degradation. It should be noted that the 70-kDa fragment can still be a good substrate for hsp70-catalyzed refolding because enzymatic activity and presumably conformation are not compromised in the 70-kDa species. Thus, topo I activity can be regenerated following heat stress from the native as well as the 70-kDa fragment of topo I in the absence of *de novo* protein synthesis. These data support the view that hsp70 proteins associate with topo I during thermal stress and suggest that this enzyme is a nuclear protein targeted for protection by hsp70 proteins. Furthermore, our analysis suggests that hsc70 and not the highly inducible hsp70 associates with topo

I under stress conditions. This view is based on the observation that a mAb specific for hsp70 (W27) did not copurify topo I, whereas N27F3-4, a mAb that recognizes hsc70 and hsp70, copurified topo I. However, we have not formally excluded the possibility that hsp70 bound to topoisomerase during stress but was dissociated from topoisomerase following binding by W27.

hsc70 is an abundant, constitutively produced cellular protein that may afford cells immediate protection from stress. *In vivo*, T cells subjected to a lethal heat shock (45°C, 30 min) still retained 20–40% of their topo I activity; this may reflect protection mediated by hsc70 (9). This view is consistent with *in vitro* protection experiments we performed using purified topo I and hsc70. Our studies demonstrated that hsc70, but not actin, protected topo I from heat inactivation. Furthermore, heat-denatured topo I could be reactivated in the presence of purified hsc70 but not actin. In none of these reactions was exogenous ATP added. Our studies with topo I are similar to the results reported for RNA polymerase (RNAP). For example, purified DnaK (the prokaryotic homologue of hsp70) protected RNAP *in vitro* from heat inactivation in an ATP-independent reaction (22). In contrast to our results, however, refolding of RNAP required ATP hydrolysis. Size-exclusion chromatography revealed that heat stress generated aggregates of RNAP ($>5 \times 10^6$ Da). The addition of DnaK and ATP resulted in the recovery of enzymatic activity as well as dissociation of RNAP aggregates. How proteins refold in the absence of ATP hydrolysis remains to be clarified. For some proteins, loss of native conformation may not result in aggregation and the need for ATP-dependent refolding. In this regard, we have been unable to detect heat-induced aggregation of topo I by HPLC size-exclusion chromatography (data not shown). Thus, our *in vitro* studies demonstrate that hsc70 can protect protein function during heat stress as well as reactive heat-denatured topo I, results that closely mirror events following heat stress *in vivo*.

It has been suggested that members of the hsp70 family are capable of "reactivating" protein function through dissolution of protein aggregates (1, 23). Although this view is consistent with refolding studies for some proteins (e.g., RNAP), studies with other aggregated proteins indicate that hsp70/ATP alone cannot dissociate protein aggregates nor restore protein function (24). For these proteins, achieving native conformation may require other cofactors. Reactivation of topo I by purified hsc70 was inefficient relative to the cell lysate, suggesting that cofactors may also be required for optimal regeneration of this enzyme. As discussed above, this view is consistent with our recent studies demonstrating that purified hsc70 became highly efficient in reactivating topo I in the presence of a cytosolic component(s). Furthermore, regeneration of topo I activity by either the lysate or hsc70 did not require ATP. Our studies with topo I suggest that the cell may possess a novel pathway to achieve native conformations that utilize hsc70 but not ATP. This possibility is consistent with studies by other investigators who demonstrated that transfection of hsp70 with a deleted ATP-binding domain still protected cells from heat stress (25).

In summary, our studies identify topo I as a nuclear protein that complexes with hsc70 during heat stress. On the basis of our *in vitro* studies, we suggest that this interaction *in vivo* limits heat-induced inactivation of the enzyme and contributes to its reactivation following heat stress. Complex formation between hsc70 and nuclear topo I may explain, at least in part, the massive influx of hsc70 into the nucleolus during heat shock. In addition, our studies suggest that an ATP-independent pathway exists for refolding proteins such as topo I that may be misfolded but not aggregated.

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