Correcting Temperature-sensitive Protein Folding Defects

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Abstract

Recently, we found that different low molecular weight compounds, all known to stabilize proteins in their native conformation, are effective in correcting the temperature-sensitive protein folding defect associated with the ΔF508 cystic fibrosis transmembrane regulator (CFTR) protein. Here we examined whether the folding of other proteins which exhibit temperature-sensitive folding defects also could be corrected via a similar strategy. Cell lines expressing temperature-sensitive mutants of the tumor suppressor protein p53, the viral oncogene protein pp60^{src}, or a ubiquitin activating enzyme E1, were incubated at the nonpermissive temperature (39.5°C) in the presence of glycerol, trimethylamine *N*-oxide or deuterated water. In each case, the cells exhibited phenotypes similar to those observed when the cells were incubated at the permissive temperature (32.5°C), indicative that the particular protein folding defect had been corrected. These observations, coupled with our earlier work and much older studies in yeast and bacteria, indicate that protein stabilizing agents are effective in vivo for correcting protein folding abnormalities. We suggest that this type of approach may prove to be useful for correcting certain protein folding abnormalities associated with human diseases. (*J. Clin. Invest.* 1997. 99:1432–1444.)
Key words: temperature-sensitive mutations • protein folding • molecular chaperones • chemical chaperones • genetic disease

Introduction

A number of low molecular weight compounds have been shown to be effective in stabilizing proteins in vitro against thermally induced denaturation (1–3). Representative compounds include polyols such as glycerol, solvents such as DMSO, and deuterated water (D_2O)[1]. In addition to their effects in vitro, some of these compounds appear to influence protein folding and/or stability in vivo (4–6). Animal cells incubated in the presence of either deuterated water or glycerol, for example, can withstand severe heat shock treatments that would otherwise be lethal to the cells. Here, addition of the compounds to the cells helps to reduce the overall extent of thermal denaturation of intracellular proteins. In yeast and bacteria, the addition of glycerol into the growth medium not only protects the cells against thermal treatments, but in some cases also is effective in correcting protein folding abnormalities due to specific mutations (7). This type of osmotic remediation has been shown to be the most effective for those mutant proteins that exhibit a temperature-sensitive protein folding defect.

Based on these earlier observations, we have been examining whether different protein stabilizing agents might be effective in correcting protein folding abnormalities associated with particular diseases (8). For example, in the majority of patients with cystic fibrosis, a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein results in the deletion of a phenylalanine residue at position 508 (ΔF508 CFTR). As a consequence, the newly synthesized ΔF508 CFTR protein fails to fold properly, and does not move to the plasma membrane where it normally functions as a cAMP-regulated chloride channel (9). Instead, the newly synthesized protein becomes trapped in the endoplasmic reticulum, likely in a complex with one or more members of the molecular chaperone family (10, 11). This folding defect of the ΔF508 CFTR protein is temperature dependent. Lowering the growth temperature (e.g., 30°C or less) of animal cells expressing the ΔF508 CFTR protein causes a portion of the mutant protein to move to the plasma membrane. These cells now appear competent for cAMP-stimulated chloride exchange, indicative of a functional CFTR protein (12–14).

We have found that the temperature-sensitive folding defect of the ΔF508 CFTR protein can be corrected when cells are cultured in the presence of different protein-stabilizing agents. For example, incubation of mouse fibroblasts expressing ΔF508 CFTR at 37°C in the presence of glycerol, trimethylamine *N*-oxide (TMAO), or deuterated water (D_2O), resulted in the proper processing of the ΔF508 CFTR protein and its subsequent transport to the cell surface. Importantly, these cells then exhibited cAMP-dependent chloride transport, similar in rate and magnitude as that observed for the cells expressing the wild-type CFTR protein (8).

Cystic fibrosis is but one of a number of genetic diseases which arise because of specific mutations that ultimately lead to protein folding errors (for reviews see reference 15 and 16). Prompted by our success with the mutant CFTR protein, we examined whether protein-stabilizing agents also might be effective for correcting other temperature-sensitive protein folding mutants. For these studies, cell lines expressing three different temperature-sensitive folding mutants were examined: (a) the tumor suppressor protein p53, (b) pp60^{src}, the transforming protein encoded by Rous sarcoma, and (c) an enzyme (E1) which catalyzes the first step in the pathway of protein ubiquitination. We show that treatment with the different pro-

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1. *Abbreviations used in this paper:* CFTR, cystic fibrosis transmembrane conductance regulator; D_2O, deuterated water; TMAO, trimethylamine *N*-oxide.

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tein-stabilizing agents resulted in the cells adopting a wild-type phenotype at the nonpermissive temperature, indicative that the particular protein folding defect had been corrected. Thus, we suspect that mutations which result in temperature-sensitive protein folding defects are amenable in general to correction in vivo via the use of protein-stabilizing agents. Furthermore, we suggest that our results may have broad implications as they relate to the correction of protein folding defects associated with certain genetic diseases.

Methods

Cell culture and indirect immunofluorescence. A1-5 cells (a generous gift from A. Levine), a cell line expressing a temperature-sensitive (ts) mutant p53 protein (ala to val change at amino acid 135), were cultured and maintained at 37°C in DMEM containing 10% fetal bovine serum. Cells expressing a ts pp60^{src} protein (kindly provided by D. T. Aftab) were routinely maintained under the same conditions. ts20 cells, which express a temperature-sensitive mutant of the E1 enzyme (a component of the ubiquitin pathway) (kindly provided by H. Ozer), were maintained at 32.5°C (permissive temperature) in DMEM medium containing 10% fetal bovine serum. For indirect immunofluorescence, cells were grown on glass coverslips. After the particular experimental treatment (described in the figure legends), cells were fixed with 100% methanol, and then subsequently rehydrated in PBS. The intracellular localization of p53 was determined by incubation of the fixed cells with the antibody 421 (Oncogene Science Inc., Mahasset, NY), which recognizes both the mutant and wild-type forms of p53. Primary antibody was visualized by subsequent staining with a rhodamine conjugated goat anti–mouse antibody. All antibodies were diluted in 5 mg/ml BSA in PBS.

Cell morphology analysis. Cells expressing the ts E1 enzyme or the ts pp60^{src} protein were incubated at either 32.5°C (permissive temperature) or at 39.5°C (nonpermissive temperature) in the presence or absence of protein-stabilization agents. Following 2 d of treatments, cells were examined by phase-contrast microscopy.

Effects of chemical treatments on cell growth. A1-5 cells were plated in 60-mm dishes at an initial concentration of 5×10^4 cells/plate. After allowing them to attach at 37°C for 16 h, the cells were shifted to the appropriate temperature in the presence or absence of the various protein stabilizing agents. A few plates of the cells were collected at the time of chemical addition, and cell number was determined using a hemocytometer. Thereafter, the cells were counted at daily intervals. All time points were examined in duplicate with the values given representing the mean for replicate counts. For ts20 experiments, cells growing at 32.5°C were plated on 60-mm dishes and maintained at this temperature for 16 h. Some of the cells were incubated with the chemicals, while the others were maintained in normal growth media. The cells were then shifted to the appropriate temperatures in the presence or absence of the chemicals.

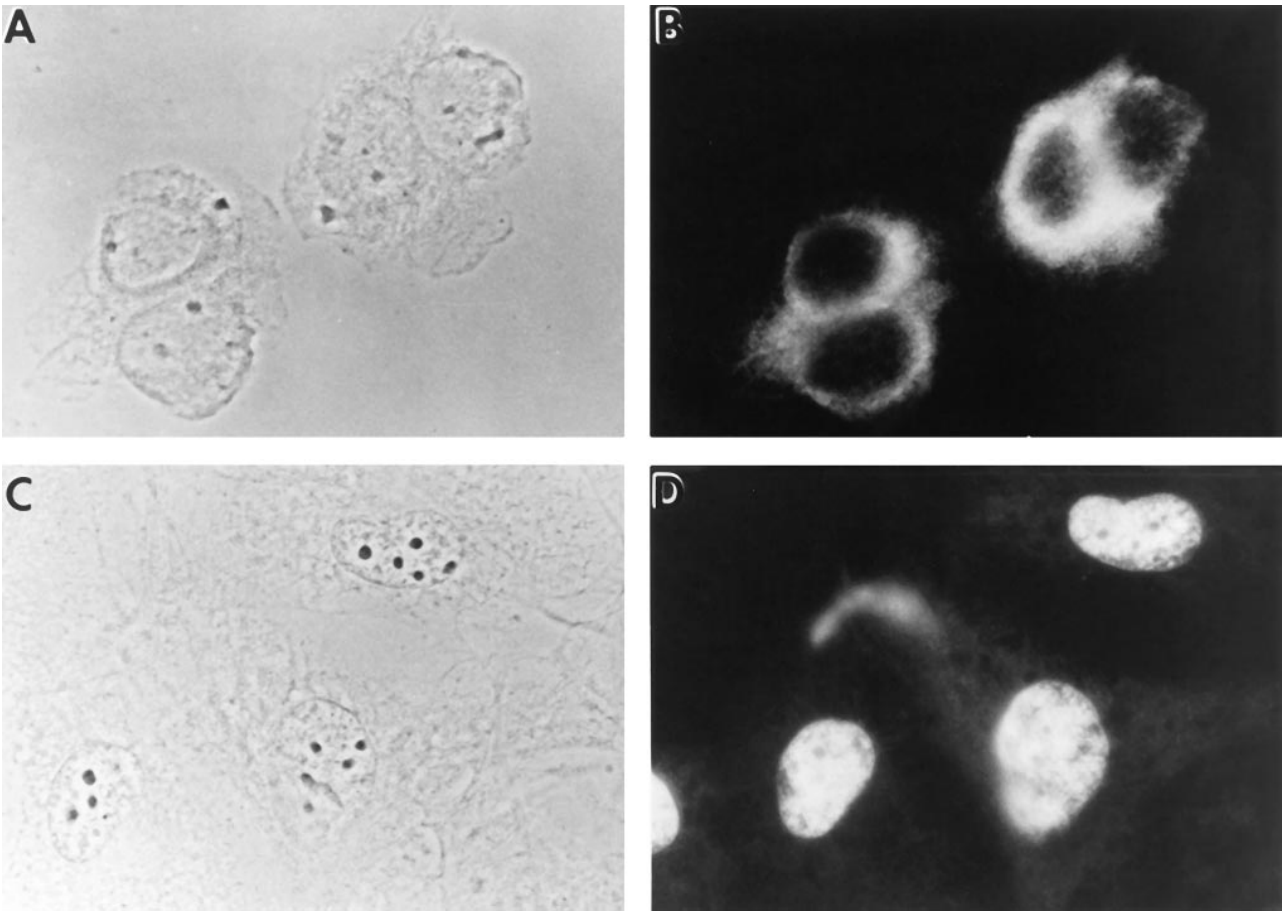


Figure 1. The intracellular localization of temperature-sensitive p53 in A1-5 cells incubated at the permissive (32.5°C) and nonpermissive (39.5°C) temperatures. A1-5 cells were grown on coverslips and incubated at either 32.5°C or 39.5°C for 2 d. The intracellular distribution of p53 was determined by indirect immunofluorescence using a monoclonal antibody which recognizes both the wild-type and mutant forms of p53 (PAb 421). *A* and *B* are the cells maintained at 39.5°C, and *C* and *D* are the cells incubated at 32.5°C. *A* and *C* are the phase contrast photographs, and *B* and *D* are the corresponding immunofluorescence photographs.

Chemical treatments. Various concentrations of protein-stabilizing agents were added to DMEM supplemented with 10% fetal bovine serum. For D_2O experiments, powdered DMEM was reconstituted using 100% D_2O (Sigma Chemical Co., St. Louis, MO) and supplemented with 10% fetal bovine serum. For each experiment, the culture medium was removed and replaced with fresh medium containing the chemical of interest. For recovery experiments (see Fig. 4) cells were incubated with the various chemicals for 2 d. The medium was then removed, and the cells were washed with and further incubated in normal growth medium not containing the particular protein stabilizing agent.

Western blotting. Following the particular experimental treatment, cells were lysed in Laemmli sample buffer and heated at 100°C for 5 min. Lysates were clarified, and the proteins were separated by SDS-PAGE. The resolved proteins were transferred to nitrocellulose and subsequently immunoblotted using the mouse monoclonal anti–p53 antibody, 421 (Oncogene Science) or the hsp73/hsp 72-specific antibody, N27 (StressGen Biotech. Corp., Victoria, Canada).

Results

Correction of a p53 protein folding mutant. A cell line that expresses a mutant form of the tumor suppressor protein p53 was chosen for our initial studies (17). This cell line (A1-5) was produced by transfection of primary rat fibroblasts with a p53 gene containing a missense mutation (ala to val at amino acid 135). As was shown previously, this mutation results in a temperature-sensitive protein folding defect (18–20). At temperatures around 32.5°C or less, the p53 protein adopts a wild-type conformation and localizes within the cell nucleus (Fig. 1 *D*). In contrast, at temperatures of 39.5°C or greater, the p53 protein fails to fold properly and does not accumulate within the nucleus. Instead, the vast majority of the mutant protein is found predominantly within the cytoplasm (Fig. 1 *B*).

We examined whether treatment of the A1-5 cells with different protein stabilizing agents would correct the temperature-sensitive protein folding defect of mutant p53. Three different reagents were examined: (a) the carbohydrate (or polyol) glycerol; (b) a methylamine, trimethylamine *N*-oxide; and finally, (c) deuterated water (D_2O). Cells were plated in normal culture medium and then incubated at 39.5 C where the p53 protein adopted the mutant conformation. The next day the culture medium was removed, and the cells were incubated in medium supplemented with either 0.6 M glycerol or 75 mM trimethylamine *N*-oxide (TMAO). In the case of the deuterated water, powdered DMEM was reconstituted with 100% D_2O, supplemented with serum, and added to the cells.

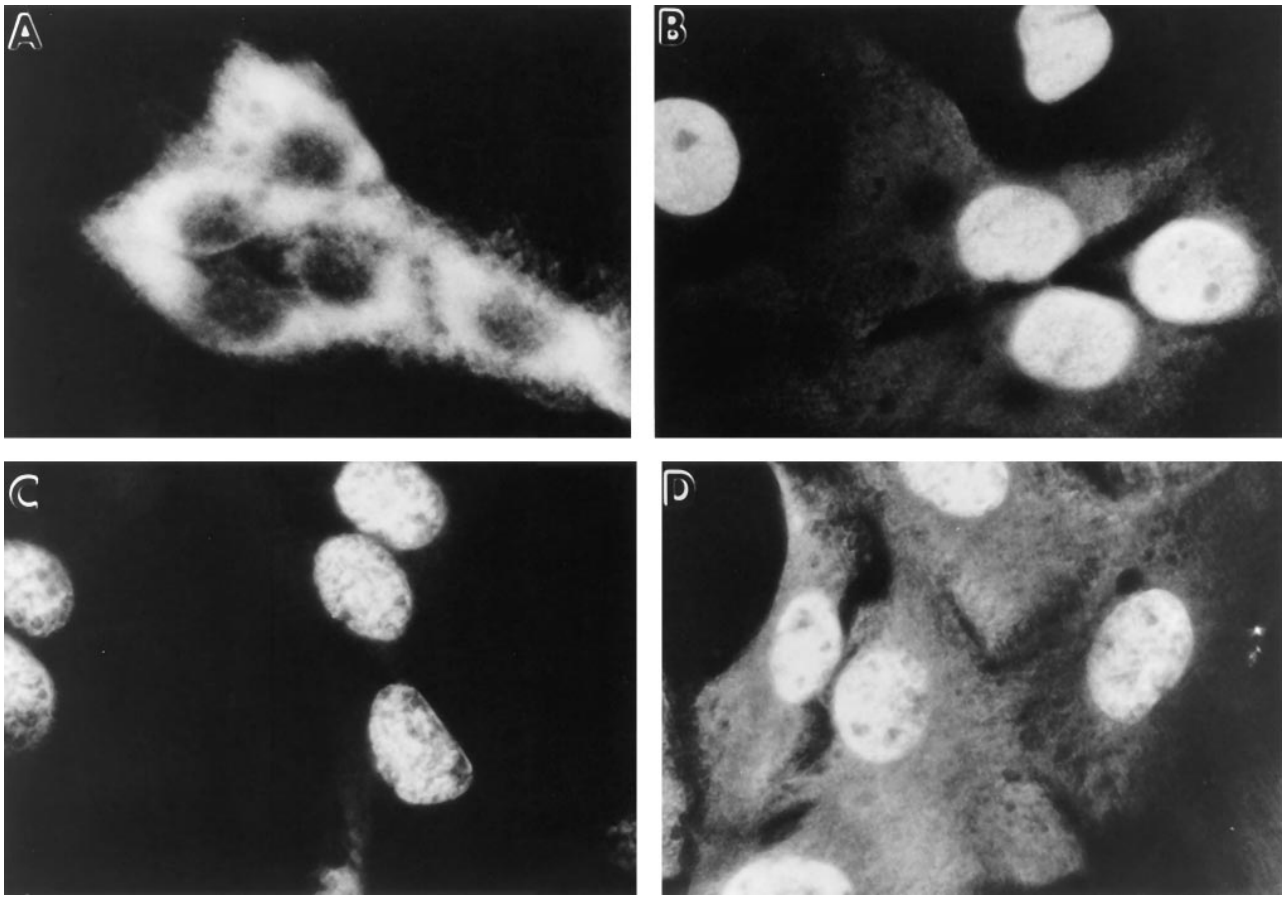


Figure 2. A1-5 cells incubated at the nonpermissive temperature in the presence of protein-stabilizing agents exhibit nuclear p53 staining. A1-5 cells were grown on glass coverslips and incubated for 1 d at 39.5°C. Some of the coverslips then were transferred into medium supplemented with either D_2O (100%), TMAO (75 mM), or glycerol (0.6 M), and the cells were further incubated at 39.5°C. After 2 d of incubation at 39.5°C, the cells were analyzed for the intracellular distribution of p53 by indirect immunofluorescence as described in Fig. 1. Fig. 2 shows only the immunofluorescent micrographs. (*A*) Control cells incubated in normal medium; (*B*) cells incubated in medium prepared with 100% D_2O; (*C*) cells incubated in the presence of 75 mM TMAO; and (*D*) cells treated with 0.6 M glycerol.

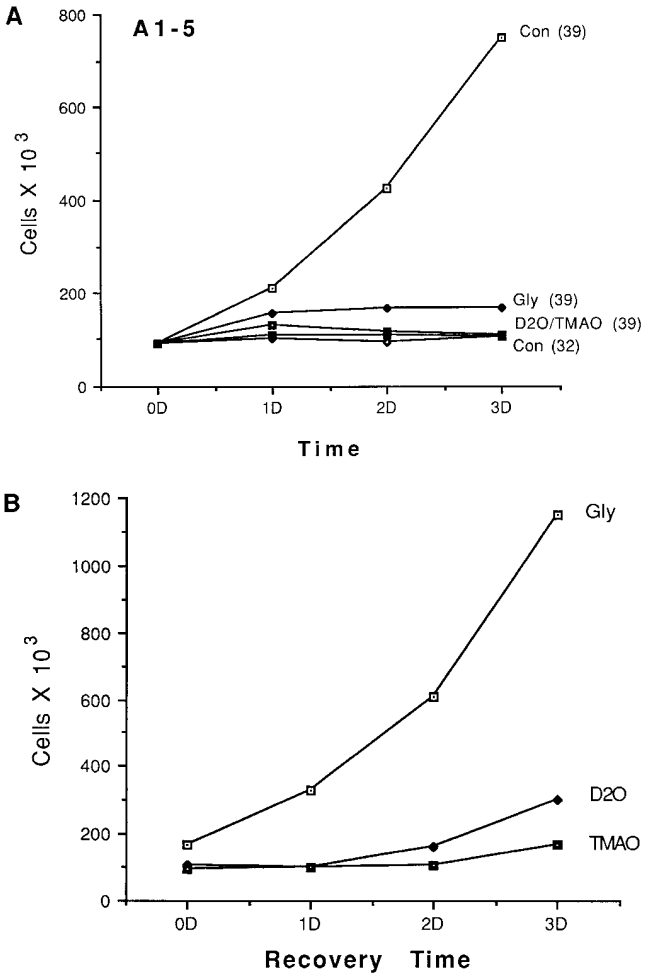


Figure 3. A1-5 cells treated with protein stabilizers at the nonpermissive temperature exhibit a wild-type phenotype as determined by cell proliferation rates. (*A*) Equal numbers of A1-5 cells, growing at 37°C, were plated on 60-mm dishes in DMEM containing 10% serum. After plating, the cells were moved to 39.5°C. The next day (day 0) the media was removed, and fresh medium supplemented with serum and containing either nothing (con), 0.6 M glycerol (Gly), 100% D_2O (D_2O), or 75 mM TMAO (TMAO), was added to the cells. The cells were further incubated at 39.5°C for 1, 2 or 3 d. After each day, a plate of the cells was removed, and cell number determined as described in the experimental methods. As a control (con [32]), one group of the cells were incubated at 32.5°C (the permissive temperature). (*B*) Equal numbers of cells were plated on 60 mm dishes and treated with the various chemicals as described above. Following 2 d of treatment, the culture medium was removed and the cells were washed extensively with (and further incubated at 39.5°C) fresh DMEM containing only 10% FBS. Cell numbers then were determined on day 1 and 2 after the changeover back into normal growth medium.

After 2 d of incubation at 39.5°C, the cells were analyzed for their distribution of p53. In the control cells maintained at 39.5°C in normal growth medium, p53 was predominantly cytoplasmic (Fig. 2 *A*). In contrast, cells incubated at 39.5°C in the presence of D_2O (Fig. 2 *B*), TMAO (Fig. 2 *C*), or glycerol (Fig. 2 *D*) now exhibited a nuclear locale of p53. Thus, incubation of the cells in the presence of the different protein-stabilizing agents appeared to result in the mutant p53 protein adopting a wild-type conformation at 39.5°C.

As an alternative method to screen for the potential beneficial effects of these compounds on p53 function, the proliferative capacity of the A1-5 cells incubated at the nonpermissive temperature (in either the absence or presence of the different chemicals) was determined. As has been shown previously (19), the A1-5 cells become growth-arrested when maintained at the permissive temperature (32.5°C), ostensibly because of the high levels of the wild-type p53 tumor suppressor protein (Fig. 3 *A*). In contrast, at 39.5°C where the overexpressed p53 protein folds incorrectly (and therefore is biologically inactive), the cells continue to proliferate normally. Hence, if treatment with the various protein-stabilizing agents corrects the folding of the mutant p53 protein, the cells now should exhibit cell cycle arrest, even when incubated at the nonpermissive temperature. Indeed, treatment of the A1-5 cells maintained at 39.5°C with any one of the three different compounds now resulted in a growth-arrested phenotype, similar to that observed for the cells incubated at the permissive temperature where the p53 protein is functional (Fig. 3 *A*).

The effects of the different protein-stabilizing agents on the phenotype of the A1-5 cells were reversible (Fig. 4). As was shown earlier, cells incubated in the presence of the different protein-stabilizing agents for 2 d at 39.5°C exhibited a nuclear locale of p53, indicative of the protein being in its wild-type conformation. When the culture medium containing the protein-stabilizing agents was removed and replaced with fresh culture medium, however, p53 again began to accumulate within the cytoplasm. For example, after only 1 d following the removal of glycerol, the cells maintained at 39.5°C no longer displayed a nuclear p53 distribution. In the case of either D_2O or TMAO, after 2 d following their removal, all of p53 now was found within the cytoplasm. Similar results were obtained when the analysis was performed by cell counts. As was shown in Fig. 3 *A*, cells incubated at 39.5°C in the presence of the three different protein-stabilizing agents for 2 d exhibited a growth-arrested phenotype, consistent with a functional p53 protein. Upon removal of the compounds and further incubation of the cells at 39.5°C in normal culture medium, however, the cells exhibited a slow resumption in their normal growth rates (Fig. 3 *B*), indicative that p53 now was in the mutant conformation.

Correcting the folding defect of pp60^{src}. The potential beneficial effects of the different protein-stabilizing agents were examined using two other temperature-sensitive mutants. Rat fibroblasts transfected with a temperature-sensitive form of pp60^{src} (the transforming protein encoded by Rous sarcoma virus) exhibit a transformed phenotype when incubated at the permissive temperature of 32.5°C (21). As shown in Fig. 5 *A*, when incubated at 32.5°C where pp60^{src} is biologically active, the cells adhere weakly to the substratum. Instead, at this permissive temperature the cells grow on top of one another to form large foci. At 39.5°C where pp60^{src} is biologically inactive, the cells display a well-spread morphology, and exhibit contact-dependent growth arrest (Fig. 5 *B*). Inclusion of glycerol into the culture medium was sufficient to restore the transformed phenotype of the cells when incubated at the nonpermissive temperature. For example, cells incubated at 39.5°C in the presence of 1 M glycerol for 3 d (Fig. 5 *C*) now exhibited a morphology very similar to the control cells maintained at 32.5°C where pp60^{src} is active (Fig. 5 *A*). Interestingly, the two other compounds, TMAO and deuterated water, were much less effective in restoring wild-

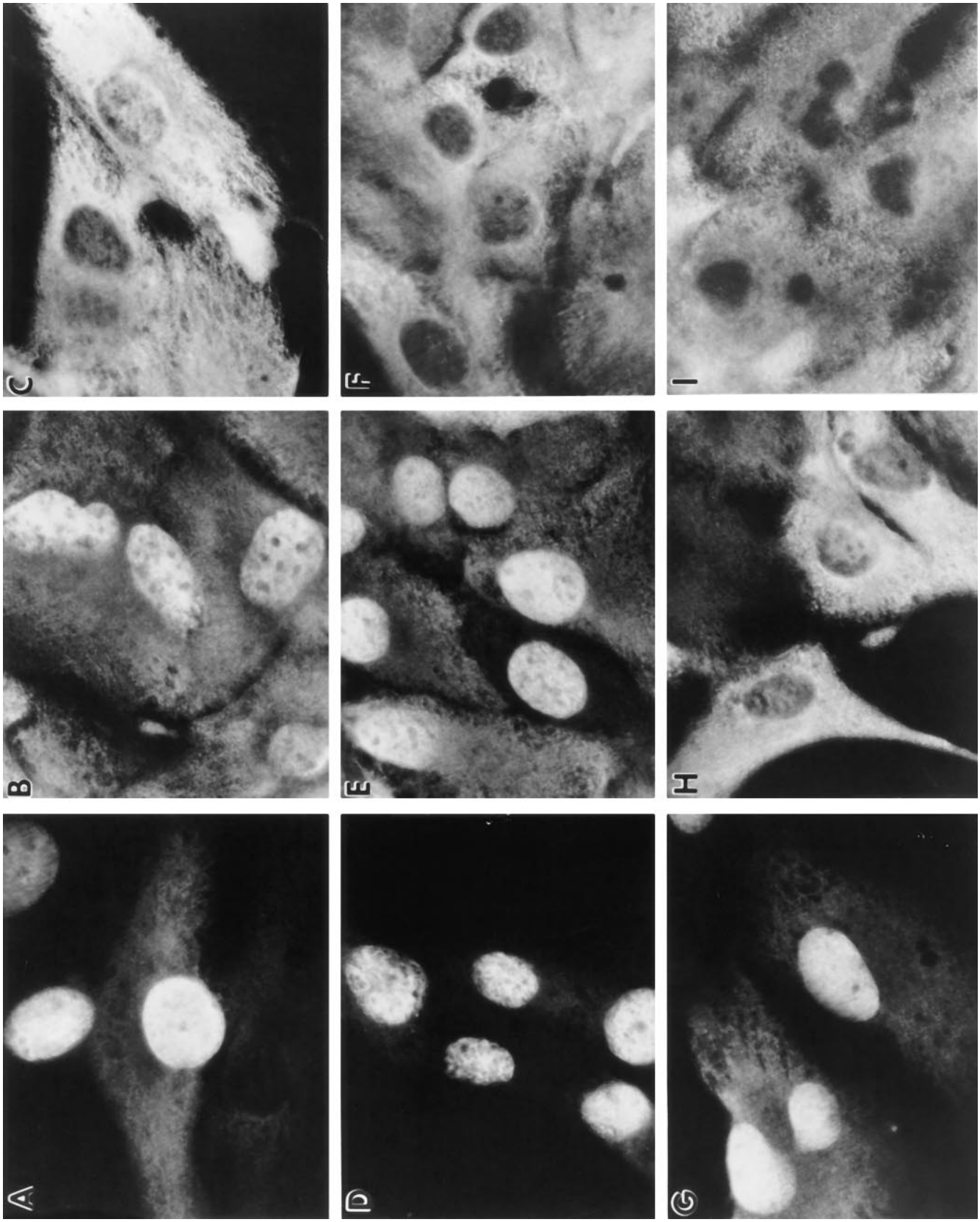


Figure 4. The effects of the protein-stabilizing agents on p53 protein folding are reversible. A1-5 cells growing on coverslips at 39.5°C were incubated in media supplemented with either; D_2O (*A–C*); TMAO (*D–F*); or glycerol (*G–I*) (concentrations as described in Fig. 3). After 2 d of incubation at 39.5°C, one coverslip from each group of cells was taken and processed for p53 indirect immunofluorescence. To the remaining coverslips, the media was removed, and the cells were washed with (and further incubated in) fresh medium supplemented only with 10% serum. Following a further 1 or 2 d incubation at 39.5°C, the cells were examined for the distribution of p53. *A–C*; cells after 0, 1, and 2 d, respectively, following removal of D_2O; *D–F*, cells after 0, 1, and 2 d, respectively, following removal of TMAO containing media; *G–I*, cells after 0, 1, and 2 d, respectively, following removal of glycerol.

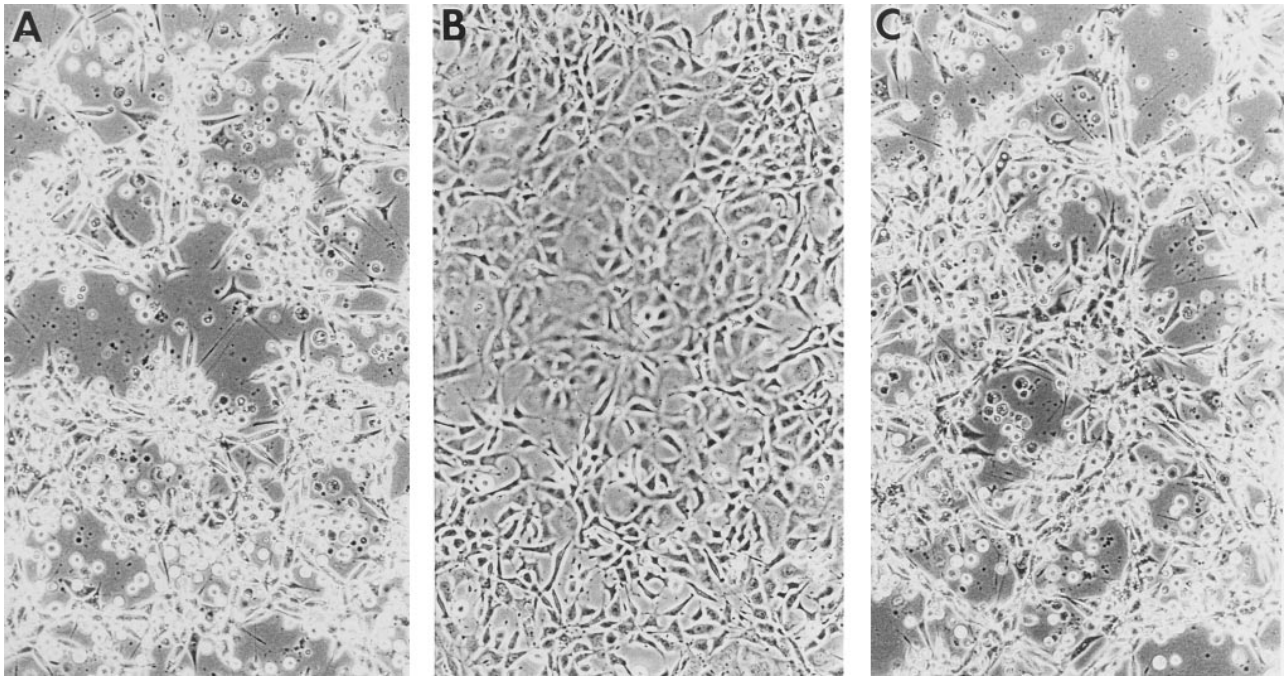


Figure 5. The presence of glycerol corrects temperature-sensitive (ts) pp60^{src} activities at the nonpermissive temperature. Cells expressing a ts form of pp60^{src} were incubated at 32.5°C, or at 39.5°C in the presence or absence of 1 M glycerol. After 3 d of treatment, the cells were examined by phase-contrast microscopy. (*A*) Cells incubated at 32.5°C for 3 d. (*B*) Cells incubated at 39.5°C for 3 d. (*C*) Cells incubated at 39.5°C for 3 d in the presence of 1 M glycerol.

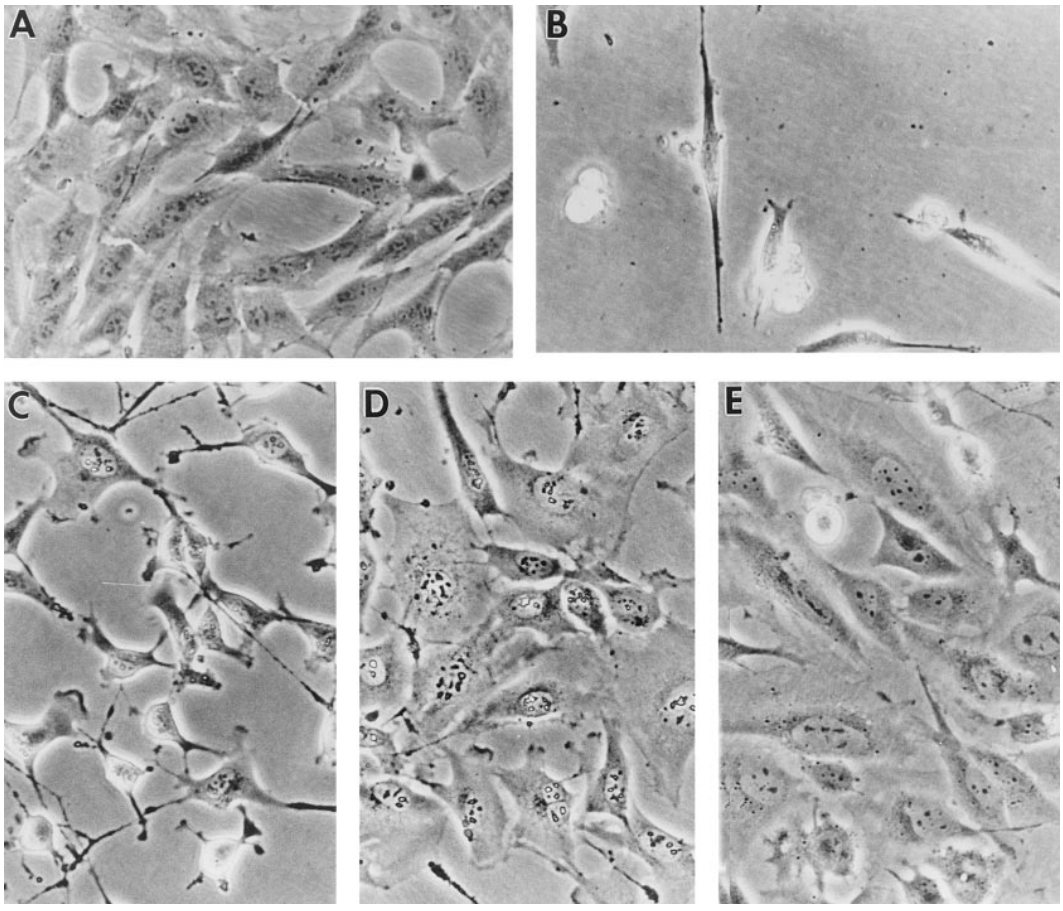


Figure 6. Effects of protein stabilizers on the morphology of ts 20 cells expressing a temperature-sensitive enzyme involved in protein ubiquitination. Equal numbers of cells expressing a temperature-sensitive mutant of the E1 enzyme were incubated at 32.5°C (permissive temp.), or at 39.5°C (nonpermissive temp.) in either the presence or absence of the different protein stabilizing agents. After 2 d of incubation the cells were examined by phase-contrast microscopy. (*A*) Cells incubated at 32.5°C. (*B*) Cells incubated at 39.5°C. (*C*) Cells incubated at 39.5°C in medium prepared with 100% D_2O. (*D*) Cells incubated at 39.5°C in the presence of 75 mM TMAO. (*E*) Cells incubated at 39.5°C in the presence of 0.75 M glycerol.

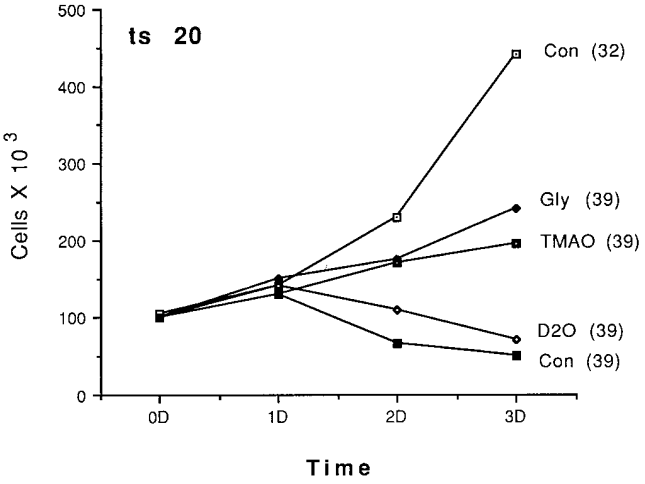


Figure 7. ts20 cells incubated in the presence of the protein stabilizers now proliferate at the nonpermissive temperature. ts20 cells were plated on 60 mm dishes at low confluence and incubated for 24 hr at 32.5°C. Control cells (no added chemicals), were then placed at either 32.5°C (permissive temp.) or 39.5°C (nonpermissive temp.). In parallel, some of the cells were incubated in the presence of the three different compounds, and then incubated at 39.5°C. After 1, 2, or 3 d of incubation, the cells were collected and cell number was determined as described in Methods. Cell number as a function of the days (D) of incubation at either 32.5°C or 39.5°C are presented.

type pp60^{src} activity at the nonpermissive temperature (data not shown).

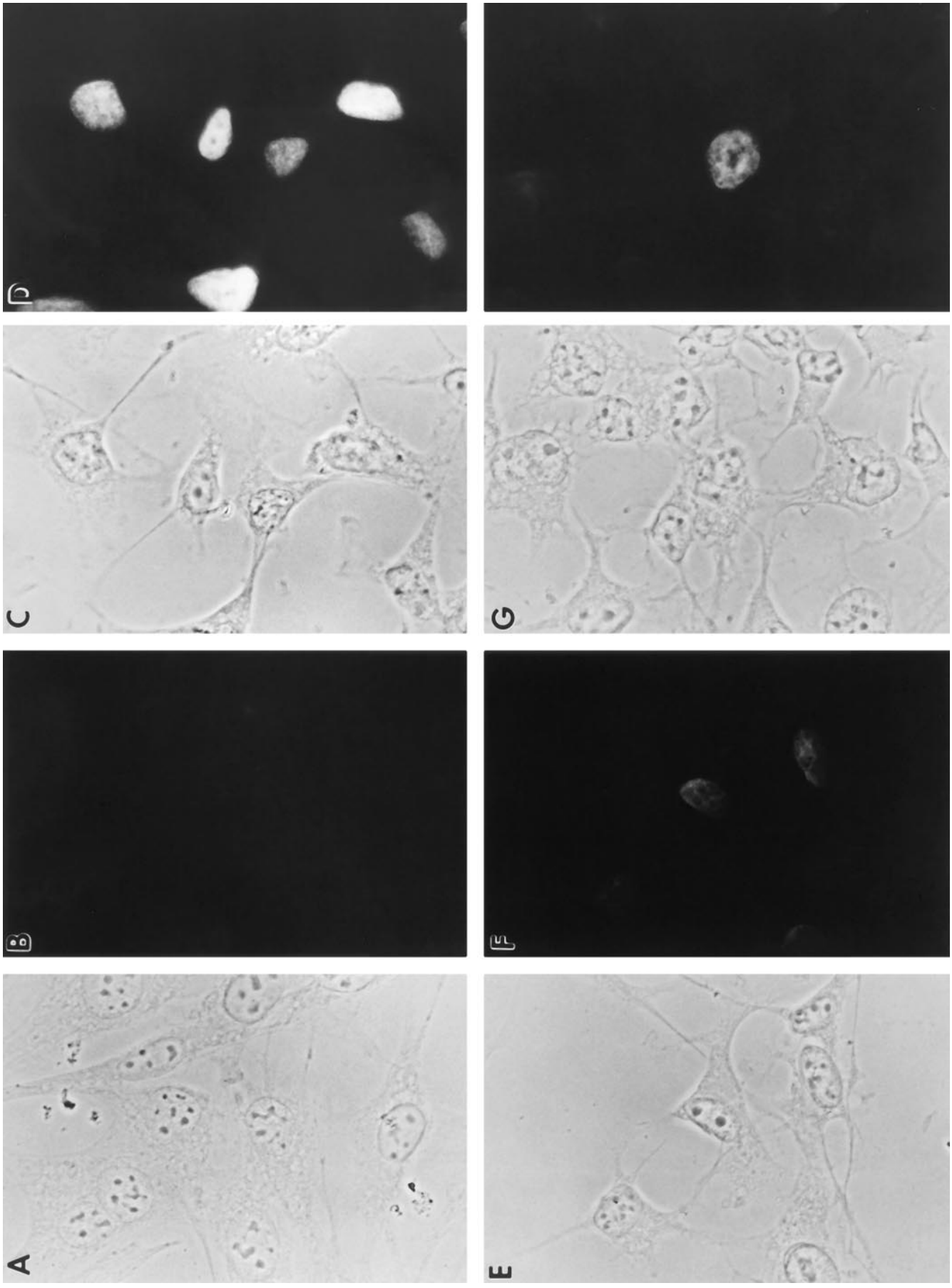
Correcting the folding defect of the E1 enzyme. The final temperature-sensitive mutant examined is an enzyme involved in the pathway of ubiquitin-dependent protein degradation. ts20 cells express a temperature-sensitive E1 enzyme that is rendered inactive at the nonpermissive temperature (39.5°C). As a consequence, these cells are unable to carry out ubiquitin-dependent protein degradation events when maintained at 39.5°C (22, 23). ts20 cells were plated at subconfluency and then incubated at 32.5°C, or 39.5°C in either the absence or presence of glycerol, TMAO, or D_2O. 2 d later the cells were examined by phase-contrast microscopy (Fig. 6). The untreated control cells maintained at 32.5°C (where the E1 enzyme is active) had grown to near confluence, and exhibited a well-spread morphology (Fig. 6 *A*). In contrast, when incubated at the nonpermissive temperature (i.e., 39.5°C where E1 is inactive) the cells did not grow at all (Fig. 6 *B*). Note that the few cells that did survive at 39.5°C exhibited a spindle-shaped morphology, markedly different than that observed for the cells grown at the permissive temperature. Interestingly, different morphological phenotypes and apparent growth rates were observed when the cells were incubated in the presence of the different protein-stabilizing agents. For example, cells incubated at 39.5°C in culture medium prepared with 100% D_2O exhibited an apparent growth rate only slightly higher than that observed for the untreated cells maintained at the nonpermissive temperature. In addition, the morphology of the cells appeared to reflect an intermediate phenotype, with both spindle-shaped and well-rounded cells being observed (Fig. 6 *C*). Treatment of the ts20 cells with either TMAO (Fig. 6 *D*) or glycerol (Fig. 6 *E*) resulted in an apparent restoration of the wild-type phenotype. Like the control cells maintained at 32.5°C, those cells incubated at 39.5°C in the presence of TMAO or glycerol exhibited normal growth rates and a well-rounded morphology.

A more quantitative comparison of the growth rates of the cells incubated at 39.5°C in the presence of the three protein-stabilizing compounds is shown in Fig. 7. Cells incubated at 32.5°C in regular culture medium displayed an exponential rate of growth, while those maintained at 39.5°C failed to grow. The actual reduction in cell number found for the cells maintained at 39.5°C was observed over the course of many experiments, and likely represents cell death (although the mechanism, necrosis versus apoptosis, remains unclear). Similar to our observations using phase-contrast microscopy, cells incubated at 39.5°C in culture medium prepared with D_2O failed to exhibit any significant growth. Cells incubated with either glycerol or TMAO at 39.5°C did exhibit a recovery of cell proliferation, albeit at rates less than that observed for the control cells incubated at 32.5°C.

Growth inhibition of the ts20 cells at the nonpermissive temperature has been suggested to be, at least in part, because of the failure of the cells to ubiquitinate and therefore degrade the normally short-lived p53 tumor suppressor protein (23). Accordingly, when the cells were maintained at the permissive temperature (at 32.5°C where E1 is functional), p53 levels were undetectable as determined by indirect immunofluorescence analysis (Fig. 8 *B*). In contrast, p53 easily was observed within the nucleus of those cells maintained at the nonpermissive temperature of 39.5°C (Fig. 8 *D*). When the cells were incubated at 39.5°C in the presence of either TMAO (Fig. 8 *F*) or glycerol, however (Fig. 8 *H*), the levels of p53 nuclear staining was significantly reduced, approaching that observed for the cells maintained at the permissive temperature (Fig. 8 *B*).

Similar results were obtained when the levels of p53 in the ts20 cells were determined by Western blotting (Fig. 9). Cells incubated at 32.5°C showed relatively low levels of p53, while those incubated at 39.5°C had much higher levels of the protein. When the cells were incubated at 39.5°C in the presence of either TMAO (T) or glycerol (G) (Fig. 9 *A*), the levels of p53 were significantly reduced, now appearing similar to those observed for the cells maintained at 32.5°C. Again, these observations are consistent with the idea that the different protein-stabilizing agents are effective in restoring a functional

Figure 8. Protein-stabilizing chemicals correct the temperature sensitive phenotype of ts20 cells as demonstrated by a lack of nuclear p53 staining at the nonpermissive temperature. ts20 cells were plated on coverslips and incubated at 32.5°C (the permissive temperature). The next day some of the coverslips were maintained at 32.5°C, while the other coverslips were placed into media either lacking or containing glycerol or TMAO, and then incubated at 39.5°C. 1 d later the coverslips were fixed, and the intracellular locale of p53 was determined by indirect immunofluorescence analysis using the monoclonal antibody PAb 421. Shown in *A*, *C*, *E* , and *G* are phase-contrast micrographs, and in *B*, *D*, *F*, and *H* are fluorescent micrographs. *A* and *B*, cells grown at 32.5°C. *C* and *D*, cells incubated at 39.5°C. *E* and *F*, cells incubated at 39.5°C in the presence of 75 mM TMAO. *G* and *H*, cells incubated in the presence of 0.75 M glycerol.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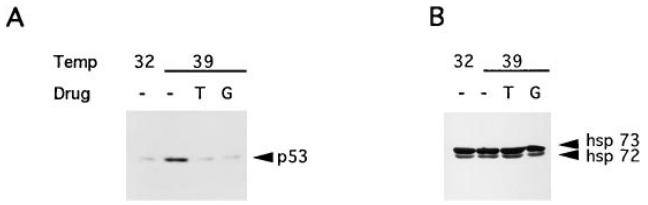

Figure 9. ts20 cells incubated at 39.5°C in the presence of protein-stabilizing agents now exhibit low levels of the p53 protein as determined by Western blotting. ts20 cells were plated on 35-mm dishes, and were incubated at 32.5°C for 1 d. One plate of cells was maintained at 32.5°C, while the other plates were incubated at 39.5°C in the absence or presence of either TMAO (T) or glycerol (G). 1 d later the cells were harvested, and the relative levels of p53 (*A*) or the cytosolic chaperones hsp73/72 (*B*) determined by Western blotting.

ubiquitin pathway (thereby resulting in normal p53 degradation), even when the cells are maintained at the nonpermissive temperature. It should be noted that the chemical treatments did not have any obvious effects on general cellular metabolism, with the overall rates of protein synthesis, for example, being similar for each treatment (data not shown). Along these lines, we were also curious to know whether incubation of the ts20 cells with any of the different protein-stabilizing agents might have an effect on the overall levels of the cytosolic hsp70 chaperones. As shown in Fig. 9 *B*, the relative levels of hsp73 and hsp72 appeared identical in the cells regardless of the incubation temperature, or the inclusion of either glycerol or TMAO into the culture medium.

We next examined whether the mutant phenotype was reversible in cells expressing the ts E1 protein. For these experiments, we again used the appearance of p53 staining (or lack thereof) as an assay for E1 enzyme activity. As shown in Fig. 10 *A*, cells maintained at 39.5°C for 2 d in the absence of protein stabilizers exhibited strong nuclear p53 staining, indicative that the E1 enzyme (and therefore the ubiquitin pathway) was inactive. Switching the 39.5°C cells back to 32.5°C for 1 d resulted in a disappearance of nuclear p53 staining (Fig. 10 *B*), indicative of a restoration of the ubiquitin-dependent protein degradation pathway. In those cells incubated at 39.5°C for 1 d in normal culture medium, and then transferred into culture medium containing either TMAO or glycerol for an additional day at 39.5°C, mixed results were obtained. Specifically, TMAO treatment resulted in a diminishment, but not complete abolition, of nuclear p53 staining (Fig. 10 *C*). In the case of glycerol treatment, however, p53 staining no longer was observed (Fig. 10 *D*).

Proper folding of the newly synthesized (but not the mature) form of the E1 protein is corrected by the chemical treatments. In our last set of experiments we attempted to determine which population of the E1 enzyme was being corrected via treatment of the cells with the different chemicals. More specifically, were the chemical treatments correcting or rescuing the misfolding of the mature (already synthesized) E1 protein, or were they only efficacious as it pertained to the folding of the newly synthesized E1 protein? To address this question, the experiments presented in Fig. 10 were repeated. This time however, the protein synthesis inhibitor cycloheximide was added before shifting the cells (on day 2) into medium containing the different chemicals. Using this approach, the mature E1 enzyme could potentially be corrected in the presence of the protein-stabilizing agents, especially considering the relatively long half-life (~20 h) of this protein (24). Instead, we observed that the addition of the protein synthesis inhibitor blocked the reversion of the cells back to the wild-type phenotype. For example, in each case, in the control cells returned to 32.5°C (Fig. 11 *B*), or the cells left at 39.5°C in the presence of either TMAO (Fig. 11 *C*) or glycerol (Fig. 11 *D*), strong nuclear staining of p53 was still observed. This is in marked contrast to the results shown in Fig. 10, where the nuclear staining of p53 no longer was observed upon temperature shift-down, or upon the addition of the different chemicals to the cells maintained at 39.5°C. Therefore, we conclude that in the case of the E1 enzyme, the different protein stabilizing agents apparently do not correct the already misfolded, mature protein. Instead, we suspect that the chemical chaperones are only effective in influencing the folding pathway of the newly synthesized E1 protein. Presumably, when synthesized at 39.5°C in the presence of the chemicals, at least a portion of the newly synthesized E1 enzyme now adopts its properly folded and biologically active conformation.

Discussion

Abnormalities in protein folding constitute the molecular basis for a number of diseases (15, 16). Oftentimes single point or deletion mutations give rise to subtle folding defects that result in either a loss of protein function, or a failure of the protein to be correctly localized. Over the past year we have been exploring new ways to affect the protein folding environment inside the cell, and thereby correct the folding of mutant proteins which fail to achieve their biologically active conformation. As we have shown here, a variety of low molecular weight compounds were effective in correcting the folding pathway of different proteins which manifest temperature-sensitive protein folding defects. These different compounds—glycerol, trimethylamine *N*-oxide, and deuterated water—all have been shown previously to be effective in stabilizing proteins against thermal or chemically-induced denaturation and aggregation in vitro (for review see reference 25). Based on their ability to stabilize proteins in vitro, as well as influence the pathway of protein folding in vivo, we now often refer to these different reagents as chemical chaperones. Like members of the protein family of molecular chaperones, the different chemical chaperones do not provide any direct information for the folding process, nor are they part of the final folded structure. Instead, they appear to influence the overall fidelity of protein folding, likely by reducing the probability of the nascent polypeptide entering into a nonproductive folding pathway.

Our previous results examining the effects of chemical chaperones on the folding of a mutant form of the cystic fibrosis transmembrane conductance regulator (CFTR) protein was the impetus for the work presented here (8). This work, as well as the studies of Sato et al. (26), showed that the temperature-sensitive folding defect associated with the ΔF508 CFTR mutant could be corrected by incubation of the cells in culture medium supplemented with the same three protein-stabilizing agents used in the present study. In yet another study, we found that the different chemical chaperones also were effective in correcting another medically important protein folding abnormality associated with neurological disease. For example, a neurological disorder referred to as scrapie or mad cow disease appears to be due to the accumulation of an abnor-

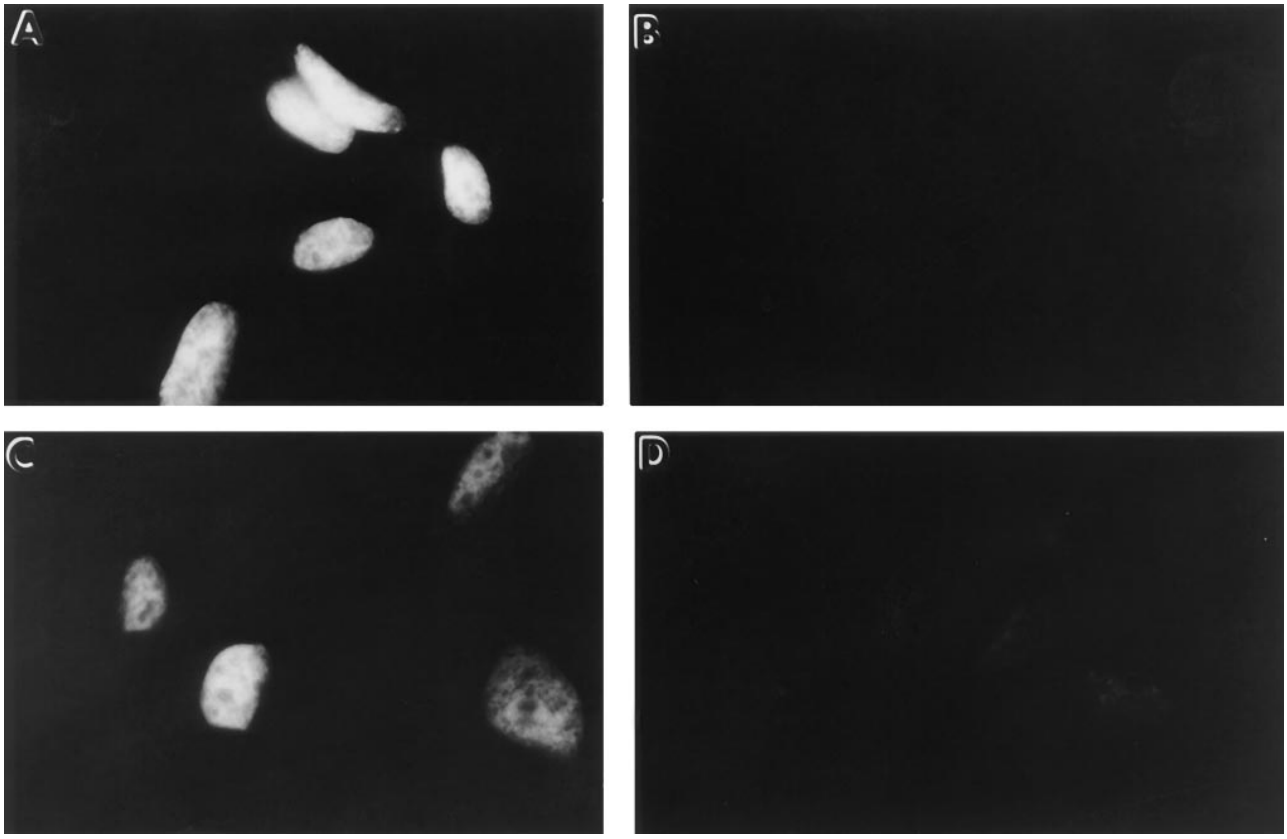

Figure 10. Addition of protein-stabilizing agents to cells already at the nonpermissive temperature still is effective in restoring wild-type E1 activities. ts20 cells were plated on coverslips and incubated for 1 d at 32.5°C. All of the coverslips then were placed at 39.5°C for 1 d to induce the mutant phenotype. The next day, fresh media containing either 0.75 M glycerol or 75 mM TMAO was added, and the cells were further incubated at 39.5°C. As a control, one of the coverslips was placed back at 32.5°C following the 1 d incubation at 39.5°C (no added chemicals). 1 d later all of the coverslips were collected, and the distribution of p53 was determined by indirect immunofluorescence as described earlier. Shown in this figure are the fluorescent micrographs. (*A*) Cells maintained at 39.5°C for 2 d. (*B*) Cells were incubated at 39.5°C for 1 d, and then placed at 32.5°C for 1 d. (*C*) Cells were incubated at 39.5°C for 1 d, TMAO was added (to a final concentration of 75 mM), and the cells were incubated further at 39.5°C for 1 d. (*D*) Cells were incubated at 39.5°C for 1 d, glycerol was added (to a final concentration of 0.75 M), and the cells were incubated further at 39.5°C for 1 d.

mally folded protein referred to as the prion protein. We have found that many of the same chemical chaperones used in the present study are effective in interfering with the formation of the pathogenic prion protein in a neuronal derived cell line (27). It is important to point out, however, that the conversion of wild-type prion into its pathogenic isoform does not appear to be a temperature-dependent process. Thus, in addition to their effectiveness for correcting temperature-sensitive protein folding defects, chemical chaperones also may prove to have efficacy in correcting other types of protein folding abnormalities.

The primary objective of the study presented here was to determine whether temperature-sensitive protein folding mutants in general could be corrected via the use of chemical chaperones. Hence, our choice of the different temperature-sensitive mutants being examined was based primarily on the ease of screening for the appearance of a wild-type–like phenotype. Owing to the results presented here, we suggest that a number of temperature-sensitive protein folding mutants can be corrected following treatment with various protein-stabilizing chemicals. Interestingly, the overall extent of correction appeared to vary somewhat as a function of the particular compound used. For example, glycerol has proven to be the most effective chemical chaperone for correcting all of the different temperature-sensitive mutants we have examined to date. High concentrations of the polyol (between 0.5-1.0 M), however, are always required to correct the particular protein folding abnormality. While the methylamine TMAO perhaps works a little less effectively than glycerol, it does so at concentrations of 100 mM or less. Finally, deuterated water has provided us with very mixed results. Although it appears to be effective in correcting some temperature-sensitive protein folding defects, oftentimes the compound can have obvious deleterious effects on the viability of the cells.

The mechanisms by which these various compounds affect protein folding or stability have been examined in vitro using defined test proteins (28, 29). Most of the different low molecular weight compounds enhance the stability of proteins to thermal or chemically induced denaturation and aggregation. In the case of glycerol (and likely other polyols), it has been suggested that the polyol tends to be excluded from the immediate vicinity of a polypeptide. Therefore, at high concentrations, glycerol might be expected to increase the relative hydration around the protein, and thereby result in the polypeptide decreasing its relative surface area. This tighter

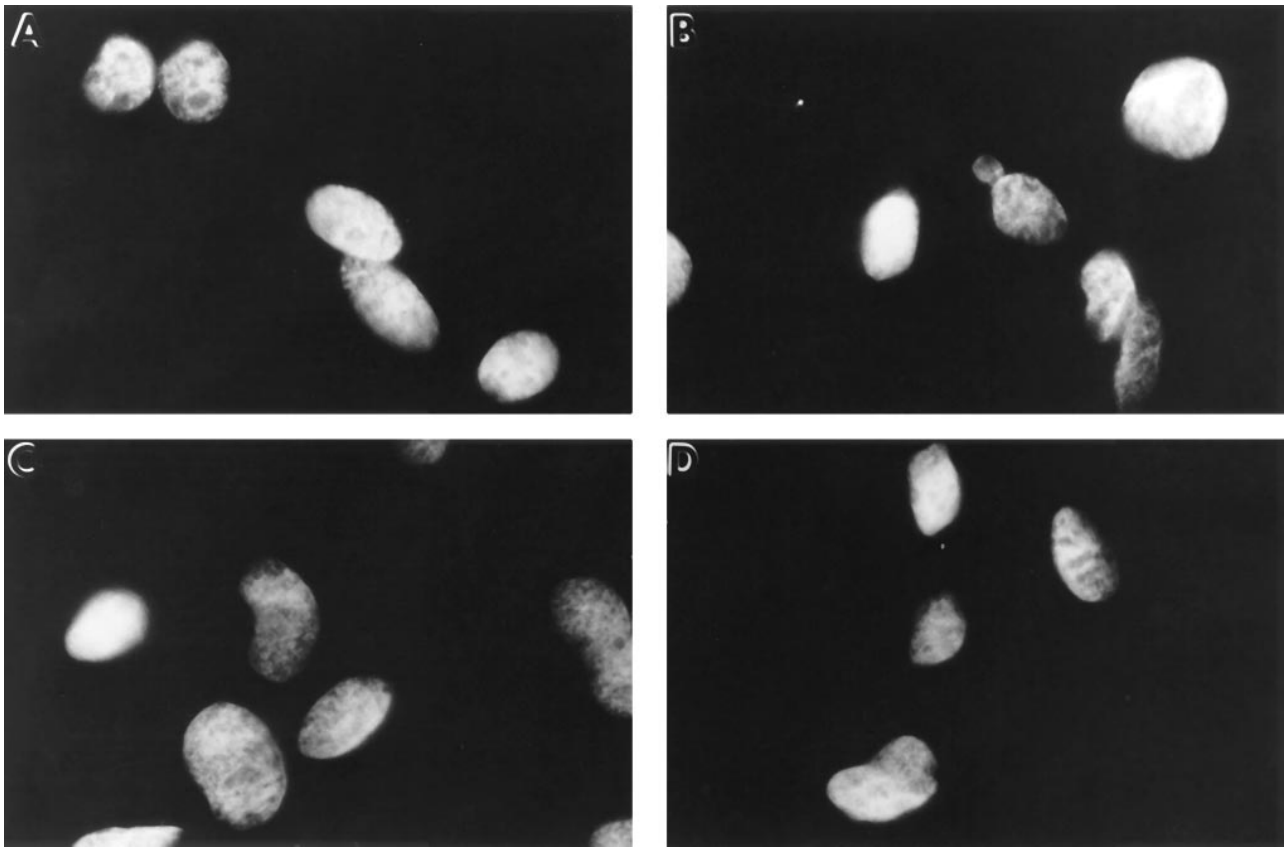

Figure 11. Protein stabilizers appear to correct the folding defect of the newly synthesized, but not the mature, form of the E1 enzyme. ts20 cells were plated on coverslips and incubated at 39.5°C for 1 d to induce the mutant phenotype. The cells were then incubated for an additional 24 h at 39.5°C with fresh media containing either 0.75 M glycerol, or 75 mM TMAO, and supplemented with the protein synthesis inhibitor cycloheximide. As a positive control, one of the coverslips incubated at 39.5°C for 1 d was returned to 32.5°C and incubated for an additional 24 h in the presence of cycloheximide. Afterwards, the cells were fixed and analyzed for the levels of p53 by indirect immunofluorescence. (*A*) Cells incubated for 1 d at 39.5°C, and then for an additional day at 39.5°C in the presence of cycloheximide. (*B*) Cells incubated for 1 d at 39.5°C, and then for an additional day at 32.5°C in the presence of cycloheximide. (*C*) Cells incubated for 1 d at 39.5°C, and then for an additional day at 39.5°C in the presence of cycloheximide and TMAO. (*D*) Cells incubated for 1 d at 39.5°C, and then for an additional day at 39.5°C in the presence of cycloheximide and glycerol.

packing of the protein, driven in a sense by an increased hydrophobic effect, would serve to enhance the stability of the protein in response to thermal or chemical treatments. Obviously, replacement of water by D_2O also would be expected to influence protein–solvent interactions, and thereby possibly affect protein folding pathways. We refer the reader to some excellent review articles which discuss in more detail the effects of these different compounds on protein folding and stability (25, 30, 31).

Two types of temperature-sensitive protein folding defects have been described in the literature: Class 1; correctly folded (mature) proteins which are destabilized upon a temperature shift, thereby resulting in their loss of activity (32); or Class 2; newly synthesized proteins which are able to fold properly at the permissive temperature, but which fold incorrectly at the nonpermissive temperature (33). All of our data indicate that it is this latter class of temperature-sensitive mutants which are being affected in our studies presented here. For example, for each of the ts mutants examined, the time needed to correct the mutant phenotype via exposure of the cells to the protein stabilizing agents was relatively long. Furthermore (at least for the E1 enzyme), in the absence of ongoing protein synthesis, inclusion of the different chemicals into the culture medium did not result in an apparent restoration of mature E1 enzyme function (albeit as determined indirectly by monitoring p53 levels). This observation, along with a number of our other unpublished findings, is consistent with the idea that it is primarily newly synthesized temperature-sensitive protein folding mutants which are amenable to correction via the chemical chaperones. We emphasize, however, that proteins do exist that manifest destabilizing temperature-sensitive mutations (e.g., Class 1 mutants), and that some of these may in fact be amenable to correction via the strategies used here (currently under study). In the case of the Class 2 mutants, we suspect that the chemical chaperones affect a critical step in the folding pathway of the newly synthesized, temperature-sensitive protein folding mutant. For example, at the nonpermissive temperature, the chemical chaperones may help in lowering a critical energy barrier which is rate-limiting in the folding pathway. In addition, the chemical chaperones might act to reduce the propensity of the newly synthesized mutant protein to go off pathway and end up in a denatured or aggregated state. Whatever

the mechanism, all of our data indicate that once the particular temperature-sensitive protein folding mutant successfully has acquired its properly folded and biologically active state, it remains correctly folded at the nonpermissive temperature, even when the chemical chaperone was subsequently removed.

The concept of using protein stabilizing agents in vivo to correct protein folding defects has been in the scientific literature for 30 yr or more. In both yeast and bacteria it was recognized that different mutations (usually missense mutations) could be corrected by either reducing the growth temperature or by increasing the osmotic strength of the growth medium. Indeed, Hawthorne and Friis in a 1964 report demonstrated that a variety of mutations in *Saccharomyces cerivisiae* could be corrected by the inclusion of compounds such as glucose, potassium chloride, glycerol, or diethylene glycol into the growth medium (7). Based on their success, they referred to their particular experimental protocol as osmotic remediation. Furthermore, in recent years it has been shown that cells from different organisms routinely employ osmotic remediation to deal with adverse changes in their environment which might lead to protein folding errors (34). For example, animal cells chronically exposed to hyperosmotic stress (e.g., cells in the kidney) respond by accumulating low molecular weight compounds in an effort to maintain their osmotic balance. These compounds, referred to as cellular osmolytes, are comprised primarily of three classes of organic compounds: carbohydrates, free amino acids (and amino acid derivatives), and methylamines. Members of the first two groups also are referred to as compatible osmolytes because of their ability to accumulate within the cell to high concentrations without significantly perturbing protein function. Alternatively, counteracting osmolytes, represented by the methylamines, are usually produced to offset the protein denaturing effects of urea. For example, in many saltwater organisms as well as in the mammalian kidney where urea can reach dangerously high levels, methylamines are found to accumulate as a way to offset the protein denaturing effects of urea (35, 36). Interestingly, at a 1:2 ratio of methylamine/urea, the potential protein denaturing effects of urea are greatly reduced.

Thus, while the concept of correcting protein folding abnormalities by changing the protein folding environment is not new, we suggest that this type of approach may represent a novel strategy to interfere with those protein folding abnormalities which constitute the molecular basis of different diseases. Based on our observations with the ΔF508 CFTR protein as well as the alanine to valine p53 tumor suppressor mutant (both of which are associated with disease in humans), we wonder how many other genetically inherited diseases result in the production of mutant proteins which exhibit temperature-sensitive folding defects. Considering the results presented here, we predict that most temperature sensitive protein folding mutants (at least of the Class 2 variety) will be amenable to correction in vivo by one or more of the chemical chaperones. Furthermore, we hope that our observations will encourage others to search for additional low molecular weight compounds which might be effective in correcting protein folding abnormalities. Identifying small molecules that are: (a) able to passively move into cells, (b) effective in influencing the protein folding environment, and (c) nontoxic to the cell tissue and organism may prove to be both faster and more efficacious for treating disease than are current approaches using gene therapy.

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References

1. Germsla, S.Y., and E.R. Stuur. 1972. The effects of combining urea and an alcohol on the heat-induced reversible denaturation of the ribonuclease. *Int. J. Pept. Protein Res.* 4:372–378.

2. Back, J.F., D. Oakenfull, and M.B. Smith. 1979. Increased thermal stability of protein in the presence of sugars and polyols. *Biochemistry.* 18:5191–5199.

3. Gekko, K., and S. Koga. 1983. Increased thermal stability of collagen in the presence of sugars and polyols. *J. Biochem. (Tokyo).* 94:199–208.

4. Lin, P.S., L. Kwock, and K. Hefter. 1981. Protection of heat induced cytotoxicity by glycerol. *J. Cell. Physiol.* 108:439–448.

5. Henle, K.J., J.W. Peck, and R. Higashikubo. 1983. Protection against heat induced cell killing by polyols in vitro. *Cancer Res.* 43:1624–1633.

6. Edington, B.V., S.A. Whelan, and L.E. Hightower. 1989. Inhibition of heat shock (stress) protein induction by deuterium oxide and glycerol: Additional support for the abnormal protein hypothesis of induction. *J. Cell. Physiol.* 139:219–228.

7. Hawthorne, D.C., and J. Friss. 1964. Osmotic remedial mutants. A new classification for nutritional mutants in yeast. *Genetics.* 50:829–839.

8. Brown, C.R., L.Q. Hong-Brown, J. Biwersi, A.S. Verkman, and W.J. Welch. 1996. Chemical chaperones correct the mutant phenotype of the ΔF508 cystic fibrosis transmembrane conductance regulator protein. *Cell Stress & Chap.* 1:117–125.

9. Cheng, S.H., R.J. Gregory, J. Marshall, S. Paul, D.W. Souza, G.A. White, C.R. O'Riordan, and A.E. Smith. 1990. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell.* 63:827–834.

10. Yang, Y., S. Janich, J.A. Cohn, and J.M. Wilson. 1993. The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp 70 and degraded in a pre-golgi non-lysosomal compartment. *Proc. Natl. Acad. Sci. USA.* 90:9480–9484.

11. Pind, S., J.R. Riordan, and D.B. Williams. 1994. Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 269:12784–12788.

12. Li, C., M. Ramjeesingh, E. Reyea, T. Jensen, X. Chang, J.M. Rommens, and C.E. Bear. 1993. The cystic fibrosis mutation (ΔF508) does not influence the chloride channel activity of CFTR. *Nature Genet.* 3:311–316.

13. Drumm, M.L., D.S. Wilkinson, L.S. Smit, R.T. Worrell, and T.V. Strong. 1991. Chloride conductance expressed by ΔF508 and other mutant CFTRs in Xenopus oocytes. *Science (Wash. DC).* 254:1797–1799.

14. Denning, G.M., M.P. Anderson, J.F. Amara, J. Marshallo, A.E. Smith, and M.J. Welsh. 1992. Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature sensitive. *Nature (Lond.).* 358:761–764.

15. Thomas, P.J., B.H. Qu, and P.L. Pedersen. 1995. Defective protein folding as a basis of human disease. *TIBS (Trends Biochem. Sci.).* 20:456–459.

16. Welch, W.J., and C.R. Brown. 1996. Influence of molecular and chemical chaperones on protein folding. *Cell Stress & Chap.* 1:109–115.

17. Finlay, C.A., P.W. Hinds, T.H. Tan, D. Eliyahu, M. Oren, and A.J. Levine. 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.* 8:531–539.

18. Michalovitz, D., O. Halvey, and M. Oren. 1990. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell.* 62:671–680.

19. Martinez, J., I. Georgoff, J. Martinez, and A.J. Levine. 1991. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. *Genes & Dev.* 5:151–159.

20. Gannon, J.V., and D.P. Lane. 1991. Protein synthesis required to anchor a mutant p53 protein which is temperature sensitive. *Nature (Lond.).* 349:802–806.

21. Maroney, A.C., S.A. Qureshi, D.A. Foster, and J.S. Brugge. 1992. Cloning and characterization of a thermolabile v-src gene for use in reversible transformation of mammalian cells. *Oncogene.* 7:1207–1214.

22. Kulka, R.G., B. Raboy, R. Schuster, H.A. Parag, G. Diamond, A. Ciechanover, and M. Marcus. 1988. A Chinese hamster cell cycle mutant arrested at G2 phase has a temperature-sensitive ubiquitin-activating enzyme, E1. *J. Biol. Chem.* 263:15726–15731.

23. Chowdary, D.R., J.J. Dermody, K.K. Jha, and H.L. Ozer. 1994. Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. *Mol. Cell. Biol.* 14:1997–2003.

24. Stephen, A.G., J.S. Trausch-Azar, A. Ciechanover, and A.L. Schwartz. 1996. The ubiquitin-activating enzyme E1 is phosphorylated and localized to

the nucleus in a cell cycle-dependent manner. *J. Biol. Chem.* 271:15608–15614.

25. Schein, C.H. 1990. Solubility as a function of protein structure and solvent components. *Bio-Technology.* 8:308–316.

26. Sato, S., C.L. Ward, M.E. Krouse, J.J. Wine, and R.R. Kopito. 1996. Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. *J. Biol. Chem.* 271:635–638.

27. Tatzelt, J., S. Pruisner, and W.J. Welch. 1996. Chemical chaperones interfere with the formation of scrapie prion proteins. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:6363–6373.

28. Gekko, K., and S.N. Timasheff. 1981. Mechanisms of protein stabilization by glycerol: Preferential hydration in glycerol-water mixtures. *Biochemistry.* 20:4667–4676.

29. Gekko, K. and S.N. Timasheff. 1981b. Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry.* 20:4677–4686.

30. Santoro, M.M., Y. Liu, S.M.A. Khan, L.X. Hou, and D.W. Bolen. 1992. Increased thermal stability of proteins in the presence of naturally occurring osmolytes. *Biochemistry.* 31:5278–5283.

31. Lin, T.Y., and S.N. Timasheff. 1994. Why do some organisms use a ureamethylamine mixture as osmolyte? Thermodynamic compensation of urea and trimethylanine N-oxide interactions with protein. *Biochemistry.* 33:12695–12701.

32. Hawkes, R., M.G. Grutter, and J. Schellman. 1984. Thermodynamic stability and point mutations of bacteriophage T4 lysozyme. *J. Mol. Biol.* 175:195–212.

33. Sturtevant, J.M., M. Yu, C. Haase-Pettingell, and J. King. 1989. Thermostability of temperature-sensitive folding mutants of the P22 tailspike protein. *J. Biol. Chem.* 264:10693–10698.

34. Burg, M.B. 1995. Molecular basis of osmotic regulation. *Am. J. Physiol.* 268:983–996.

35. Yancey, P.H., M.E. Clark, S.C. Hand, R.D. Bowlus, and G.N. Somero. 1982. Living with water stress; evolultion of osmolyte systems. *Science (Wash. DC).* 217:1214–1222.

36. Somero, G. 1986. Protons, osmolytes and fitness of internal milieu for protein function. *Am. J. Physiol.* 251:197–213.