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Depletion of Topoisomerase II in Isolated Nuclei during a Glucose-Regulated Stress Response

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Conditions, such as anoxia or glucose starvation, which induce the glucose-regulated set of stress proteins also lead to resistance to adriamycin (J. Shen, C. Hughes, C. Chao, J. Cai, C. Bartels, T. Gessner, and J. Subjeck, Proc. Natl. Acad. Sci. USA 84:3278–3282, 1987) and etoposide. We report here that chronic anoxia, glucose starvation, 2-deoxyglucose, the calcium ionophore A23187, glucosamine, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and tunicamycin (all specific inducers of the glucose regulated system) lead to a rapid and selective depletion of topoisomerase II from isolated nuclei of Chinese hamster ovary cells. This effect precedes a decline in tritiated thymidine incorporation and a redistribution of cells from S into G_1/G_0. The depletion of the enzyme is not accompanied by a decline in mRNA levels. We have also examined the mutant Chinese hamster K12 cell line which is temperature sensitive for expression of glucose-regulated proteins. When nuclei were isolated from K12 cells incubated at the nonpermissive temperature, a loss of topoisomerase II was again observed in congruence with the expression of stress proteins and cellular resistance to etoposide. These changes were not obtained in parental Wg1A cells incubated at the same temperature. These studies indicate that topoisomerase II is highly sensitive to glucose-regulated stresses and that its depletion from the nucleus, with the associated changes in cell cycle parameters, may represent general characteristics of the glucose-regulated state. Since anoxia and glucose starvation can occur during tumor development, this pathway for expression of drug resistance may have clinical ramifications.

Glucose-regulated proteins (grps) belong to a class of ubiquitous stress proteins which also include the heat shock proteins. Although the heat shock response is conserved throughout evolution, the glucose-regulated proteins are not heat inducible (48), and to date a glucose-regulated response has been studied in higher organisms only. The expression of grps is sensitive to several biologically relevant conditions such as glucose starvation (26), anoxia (31), low pH (50), changes in intracellular calcium levels (27, 49), and a variety of inhibitors of glycosylation (48). Indeed, interference in protein glycosylation leading to the presence of abnormally processed proteins in the endoplasmic reticulum is believed to be a common factor of many grp inducers (9, 16, 25). However, it is not clear how changes in the endoplasmic reticulum are transmitted to the nucleus, leading to the expression of grp genes, and, to date, the effect of various inducers of grps on nuclear composition and/or function has not been examined. The present report describes major nuclear changes which are associated with inducers of the glucose-regulated response.

Heat shock proteins are associated with the protective function known as thermotolerance (35, 36). Correspondingly, we have considered the possibility that grps also express a protective role and have previously demonstrated that various grp inducers protect cells from adriamycin cytotoxicity (33). Despite the capacity of the grp-inducing stresses to induce drug resistance, the kinetics of expression of this resistance did not agree with increased grp levels obtained in stressed cells. However, a correlation was obtained between adriamycin resistance and the entrance of the cell into and its recovery from a glucose-regulated state, as determined by the induction and repression of this class of stress proteins (33).

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Adriamycin is considered to have several modes of action in the cell (3, 4, 19, 20, 42, 44). One prominent target for adriamycin and certain other drugs is the nuclear enzyme topoisomerase II (28, 38, 42). Under normal conditions topoisomerase II covalently binds to DNA, reversibly cutting double-stranded DNA and allowing strand passage (1, 24). Several studies indicate that topoisomerase II acts as an important intermediary in the cytotoxic action of adriamycin and various other DNA-interactive drugs, including 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide, mitoxantrone, etoposide, and teniposide (reviewed in reference 28). These drugs stabilize the covalent topoisomerase II-DNA complex, preventing the rejoining of the DNA strand and leading to irreversible DNA scission. Topoisomerase II levels (and activity) decline in slowly proliferating and quiescent cells, an effect which generally correlates with reduced sensitivity to these drugs (6, 37, 38).

In the present study we analyzed several stresses, all specific for the induction of grps, and the Chinese hamster K12 temperature-sensitive cell line which is recognized to induce grps at the nonpermissive temperature (17). It is demonstrated that the induction of grps in each case leads to resistance to etoposide, a topoisomerase II-targeted agent, as the apparent result of a concomitant decline in nuclear levels of this enzyme. The reduction in topoisomerase II levels is paralleled by a reduction in etoposide-induced DNA strand breaks and precedes a decline in DNA synthesis and a redistribution of cells into a G_1/G_0 state. These observations suggest that major nuclear alterations accompany the entrance of a cell into a glucose-regulated state and that the resistance obtained during the induction of grps is caused, at least in part, by a reduction in the level of this principal target of action of an important category of antineoplastic agents.

MATERIALS AND METHODS

Cells. Cells of the line CHO (chinese hamster ovary), initially obtained from R. Tobey, Los Alamos National Laboratory were cultured in Ham F-10 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% newborn calf serum. Other special media used (e.g., methionine-deficient or glucose-free medium) were obtained from the same supplier. Chinese hamster Wg1A and temperature-sensitive mutant K12 cell lines were obtained from Amy S. Lee, University of Southern California, and were cultured as described previously (17). Briefly, these cells were cultured in Dulbecco modified Eagle medium containing 10% cadet calf serum. The permissive temperature was 35°C, and the nonpermissive temperature was 40.5°C.

Protein radiolabeling and Western immunoblot analysis. [^{35}S]methionine (>800 Ci/mmol) was obtained from Amersham International Ltd., Amersham, England, and added to methionine-free media at a final level of 10 μCi/ml for use in pulse-labeling experiments. Following stress, cells were labeled, washed twice with media without serum at 4°C, suspended in Hanks balanced salt solution without calcium and magnesium and containing 1 mM tosyl-L-arginine methyl ester, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 mM EDTA (Sigma Chemical Co., St. Louis, Mo.), and sonicated at 4°C. Protein determinations (for gel loading) were made on lysate by using the method recommended by Bio-Rad Laboratories, Richmond, Calif., and fourfold-concentrated sodium dodecyl sulfate sample buffer was then added to 3 volumes. A discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) system was used (14). Gels were stained (and exposed to XAR-5 X-ray film; Eastman Kodak Co., Rochester, N.Y.) or the proteins were transferred to nitrocellulose filters. In the latter case, the gels were stained with Coomassie brilliant blue after the transfer to confirm the uniformity of the transfer. The filters were incubated overnight at room temperature with a 1:500 dilution of the rabbit antibody. The immunoblots were counterreacted with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described previously (43). The quantity of topoisomerase II was obtained by photographing the blot and scanning the negative by using a Quickscan scanning densitometer (Helena Laboratories, Beaumont, Tex.). The area under the peak was calculated by using the base × height formula. All areas were then normalized to the control level. The linearity of the procedure was verified by using dilutions of control nuclei, and a reproducibility of approximately ±10% was obtained.

Induction of grps. Details of procedures for application of various grp-inducing stresses are described by Shen et al. (33). Briefly, for glucose starvation, glucose-free F-10 media with full serum were used. The glucose level was then monitored (Glucose colorimetric assay; Sigma), and cells were allowed to deplete the residual glucose in the serum. In the experiments presented here, zero time reflects the time at which glucose was no longer detectable (approximately 24 h in the above-mentioned media). Anoxia was achieved by placing flasks in a sealed Brewer jar (BBL Microbiology Systems, Cockeysville, Md.) (31, 33), and anaerobiosis was initiated by using a disposable hydrogen generator (GasPak; BBL Microbiology Systems) which creates a 5% CO_2 atmosphere. Incubations in the jar were carried out in a hot room (37°C). Anoxia was verified by using a methylene blue indicator. Chemical inducers of grps were applied at the concentrations predetermined to lead to a strong induction of grps and result in minimal or no lethality. These levels were 1 mM EGTA, 1.0 μg of tunicamycin per ml, 10 mM glucosamine, 10 μM A23187, and 10 mM 2-deoxyglucose. Nuclear fractions were obtained at various times after exposure to each of these conditions.

Nuclear and nuclear matrix isolation. Cells (4×10^7) were collected, rinsed, and centrifuged at $300 \times g$ for 5 min at 0°C. The pellet was then suspended for 15 min in 2 ml of cold lysis buffer containing 10 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, 10 mM NaCl, and 1 mM PMSF; 200 μl of 10% Nonidet P-40 was then added, and the cells were left on ice for 5 min. The sample was then homogenized with a Dounce pestle homogenizer (20 strokes) before being centrifuged at $300 \times g$ for 10 min at 0°C. The nuclear pellet was suspended in 2 ml of 50 mM Tris (pH 7.5)–3 mM $MgCl_2$–25 mM KCl–2 mM $CaCl_2$–1 mM PMSF–0.25 M sucrose, overlaid on the same buffer containing 0.6 M sucrose, and spun at $2,000 \times g$ for 10 min at 0°C. The pellet was washed with 2 ml of 50 mM Tris (pH 7.5)–25 mM KCl–5 mM $MgCl_2$–1 mM PMSF–0.25 M sucrose, overlaid again on the same buffer containing 0.6 M sucrose, and again centrifuged at $2,000 \times g$ for 10 min at 0°C. For analysis of topoisomerase II levels, isolated nuclei were suspended in 0.3 ml of 20 mM Tris–5 mM $MgCl_2$–1 mM PMSF and sonicated for 10 s. DNase I was added to a final concentration of 5 μg/ml. Following incubation at 23°C for 10 min, samples were mixed with gel electrophoresis sample buffer and boiled in preparation for electrophoresis. The resultant gels were stained, exposed to X-ray film, or transferred to nitrocellulose filters for reaction with antisera. Topoisomerase II was directly visible by Coomassie blue staining, and its disappearance can be observed by this method. The matrix fraction was obtained by digesting the washed nuclei with DNase I in the above buffer at 23°C for 15 min and centrifuged at $6,000 \times g$ for 10 min. The pellet was extracted with 1 M NaCl in 50 mM Tris (pH 7.5)–5 mM $MgCl_2$ at 4°C and centrifuged at $10,000 \times g$ for 10 min.

Northern (RNA) blot analysis. The amount of topoisomerase II mRNA in CHO cells was determined by using the cDNA clone which was derived from the human topoisomerase II gene of HeLa cells (45) (kindly provided by L. Liu, Johns Hopkins University). mRNA was extracted from exponentially growing CHO cells following various periods of stress by using the method of Chomczynski and Sacchi (5). RNA (20 μg) was denatured with glyoxal and dimethyl sulfoxide, electrophoresed on a 1% agarose gel, and transferred to nitrocellulose paper. The filters were then probed with nick-translated human topoisomerase II cDNA (1×10^6 cpm/ml; 3×10^8 cpm/μg). After 45 h of hybridization at 42°C, the filters were washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 50°C for 1 h. The filters were then dried and exposed to preflashed Kodak XAR X-ray film for 4 days to quantitate to mRNA. The filters were then probed with a nick-translated 18S ribosomal cDNA probe and exposed to preflashed X-ray film for 1 h.

Flow cytometry and tritiated thymidine incorporation. Cell cycle analysis of CHO cells following exposure to grp inducers was performed by previously published methods (37). DNA synthesis was quantitated by using [^{3}H]thymidine incorporation and scintillation spectrophotometry. Briefly, cells were labeled with 10 μCi of [^{3}H]thymidine per ml for 30 min, rinsed with F-10 medium (containing unlabeled thymidine) at 4°C, and trypsinized. Equivalent numbers of cells for each sample were deposited on Filtermats (Skatron, Inc., Sterling, Va.), rinsed, and counted.

Alkaline elution. Drug-induced DNA strand scission was

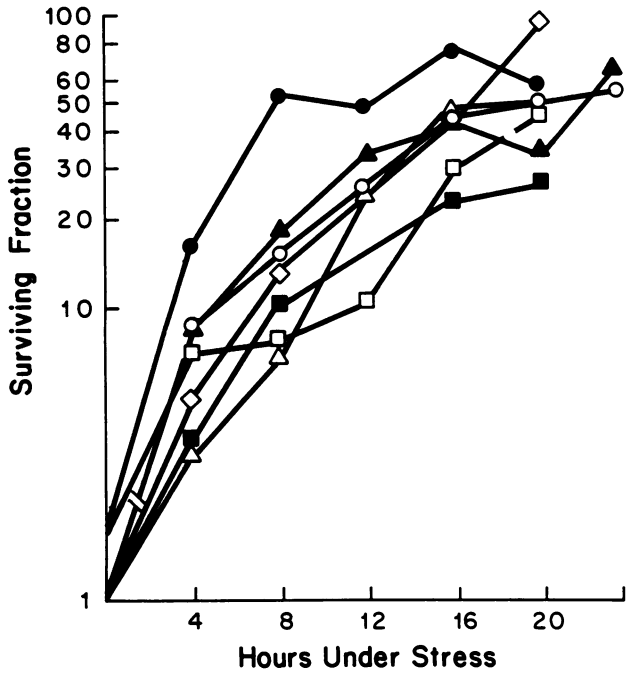

FIG. 1. Survival response of CHO cells to etoposide. CHO cells were treated with grp inducers for the indicated periods before being challenged with 30 μM VP-16 for 1 h. The cells were then washed, and survival was determined by a colony formation assay. The inducers were glucose starvation (○), anoxia (▲), 10 mM 2-deoxyglucose (●), 10 μM A23187 (□), 10 mM glucosamine (■), 1 mM EGTA (△), or 1 μg of tunicamycin per ml (◇). Datum points are means of two or three experiments.

measured by using the alkaline elution technique. Strand break frequency was quantitated as rad equivalents (52).

RESULTS

Colony survival studies. Seven specific inducers of the glucose-regulated set of stress proteins (i.e., they do not coinduce the heat shock system) were used in this study. In each case the concentrations and times used were chosen to lead to a strong induction of grps and be essentially nonlethal. After 12 h of exposure none of the stresses reduced colony survival, and after 24 h the reduction in survival was, at most, 40% (obtained with anoxia), with other stresses producing either less significant or no changes in colony-forming ability. At increasing times after the application of each agent, cells were exposed to 30 μM etoposide for 1 h to measure their survival response to the drug. From the results of these experiments (Fig. 1), it is clear that all the glucose-regulated stresses tested lead to a rapid expression of etoposide tolerance. These data compare well with those from earlier studies with adriamycin (33).

These studies with stress protein inducers were supplemented by using the K12 temperature-sensitive mutant cell line isolated from Chinese hamster fibroblasts. This cell line overproduces grps when incubated at the nonpermissive temperature as a result of a defect in protein glycosylation (17). When the temperature was changed from 35 to 40.5°C, an enhanced level of synthesis of grps was obtained within the first 4 h of incubation, after which their level of synthesis increased further, plateauing after 12 h. Incubation at the nonpermissive temperature did not lead to a heat shock response in these cells (as determined by a protein synthesis analysis). When the cells were challenged with 30 μM etoposide for 1 h at increasing times after the temperature shift, a substantial increase in resistance to etoposide was observed in approximate congruence with the induction of stress proteins (Table 1). When a similar study was performed with parental Wg1A cells, which do not induce a glucose-regulated response at the same temperature, no expression of resistance was obtained (Table 1). Wg1A cells are, however, more sensitive to etoposide at the permissive temperature, suggesting that these cell lines may differ in other respects. However, the specificity of the response of the K12 cells, relative to the Wg1A cells, after the shift to 40.5°C suggests that etoposide resistance is an aspect of the temperature-sensitive nature of the mutant K12 line. Thus, independent of whether the glucose-regulated response was activated through the application of an external stress or through a temperature-sensitive mutation, a state of drug resistance was obtained.

TABLE 1. Etoposide sensitivity at increasing times at the nonpermissive temperature in Wg1A and K12 Chinese hamster cells

Time at 40.5°C (h)	% Survival (plating efficiency) of[a]:	
	Wg1A	K12
0	0.20 (47)	1.0 (57)
4	0.01 (48)	2.4 (47)
8	0.01 (48)	6.4 (54)
12	0.03 (29)	16.3 (42)
16	0.12 (22)	48.1 (32)

[a] Data represent the mean of two or three independent experiments. Since continued exposure at 40.5°C alone affected survival, plating efficiencies were also obtained at each time (as listed) and are included in each final survival value presented here.

Protein and immunoblot analysis of stressed cells. Figure 2 presents a characteristic total protein profile of cytoplasmic and nuclear extracts of CHO cells treated for the indicated times with 10 mM glucosamine. The principal alteration in the cytoplasmic fraction appears as the induction of grps (Fig. 2A, arrows), whereas the disappearance of a 180-kilodalton (kDa) protein from the nuclear fraction was observed (Fig. 2B, arrow). An additional aspect of the disappearance of this 180-kDa protein from the nuclear fraction was its apparent specificity by gel electrophoresis analysis among other nuclear proteins, the quantities of which were unaltered (as seen by Coomassie blue staining) during grp induction. Also, the mass of total protein in various cellular compartments was essentially unaffected by treatment (i.e., the depletion of the 180-kDa protein was not a reflection of a reduction in total nuclear protein as a result of starvation, etc.). The effect of 6 and 12 h of 2-deoxyglucose treatment on the protein content of cell fractions was as follows (in the order 0 h [control], 6 h, and 12 h of 2-deoxyglucose): cytosol, 102, 96, and 113 $\mu g/10^6$ cells; high-salt nuclear extractable, 20, 17, and 19 $\mu g/10^6$ cells; and high-salt nuclear insoluble, 14, 18, and 17 $\mu g/10^6$ cells. Thus, the disappearance of the 180-kDa protein was not explained by a nonspecific loss or degradation of nuclear protein, but was a selective response.

Since the mechanism of action of etoposide is associated with the activity of topoisomerase II, which also has a molecular size of approximately 180 kDa (as does the protein just described), the effect of each of these glucose-regulated stresses on the nuclear levels of this protein was examined. Figure 3 presents typical Western blots of the nuclear fraction of CHO cells following increasing times of exposure to anoxia, 2-deoxyglucose, glucosamine, or glucose starvation (Fig. 3A to D, respectively) by using an rabbit antibody against topoisomerase II. The antisera reacted with the

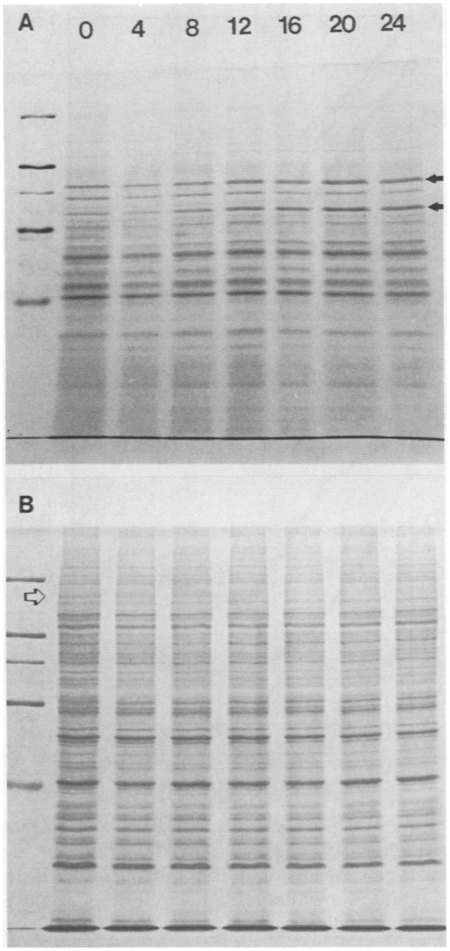

FIG. 2. Total protein profile of CHO cells. CHO cells were treated with 10 mM glucosamine for the times indicated and fractionated, and the fractions were analyzed by gel electrophoresis. The induction of grps is clearly shown in the cytosol (A), and a decline in the level of a 180-kDa nuclear protein is seen in the lower panel (B).

180-kDa band as indicated by coincidence of (i) migration and (ii) the decline in protein levels obtained in stressed cells. The immunochemical data presented in Fig. 3 are shown in quantitative form in Fig. 4 (normalized to control levels of topoisomerase II). The depletion of topoisomerase II correlates reasonably with the development of drug resistance observed in Fig. 1. The other conditions examined in Fig. 1 (i.e., EGTA, A23187, and tunicamycin) lead to analogous data when analyzed at 12 h (data not shown). However, it should also be noted that some inducers (e.g., 2-deoxyglucose and glucosamine) leave substantial residual topoisomerase II levels relative to anoxia, although comparable levels of resistance are obtained (Fig. 1 and 4). This would suggest that these stresses have differing secondary effects (e.g., on drug uptake [33]) which also affect survival.

These data were supplemented by analysis of topoisomerase II levels in the K12 temperature-sensitive mutant cell line and the parental Wg1A line. A rapid decline in topoisomerase II levels was obtained after shifting K12 cells to the nonpermissive temperature (Fig. 5); this effect was not seen with the wild-type Wg1A cells after the same temperature shift. Thus, there is a strong correlation between the entrance of a cell into a glucose-regulated state, a decline in cellular levels of topoisomerase II, and resistance to etoposide.

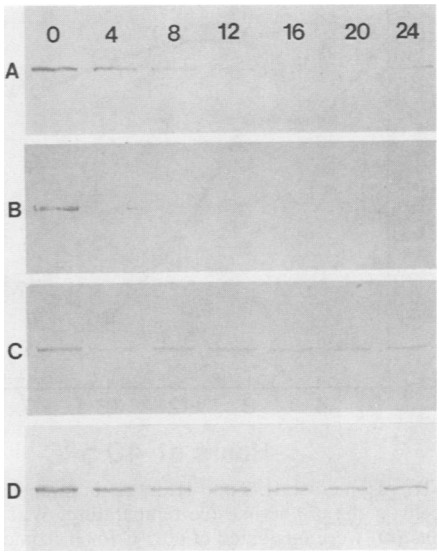

FIG. 3. Western blots of nuclear fraction of CHO cells. CHO cells were treated by anoxia (A), by glucose deprivation (B), with 10 mM 2-deoxyglucose (C), or with 10 mM glucosamine (D) for the times indicated. The cells were then fractionated, and the nuclear fractions were analyzed by Western blotting with a rabbit antibody against topoisomerase II. A decline in the topoisomerase II level was obtained during each stress.

Northern blot analysis. To more fully characterize the basis for the loss of topoisomerase II following cellular stress, we assayed the topoisomerase II mRNA content after various periods of exposure to 10 mM 2-deoxyglucose. There was no discernible decrease in mRNA after 6 h of treatment (Fig. 6). After this time point, however, there was a progressive loss of mRNA, and by 18 h it was virtually undetectable. Similar results were obtained when 10 mM glucosamine was used as the cellular stress. In contrast,

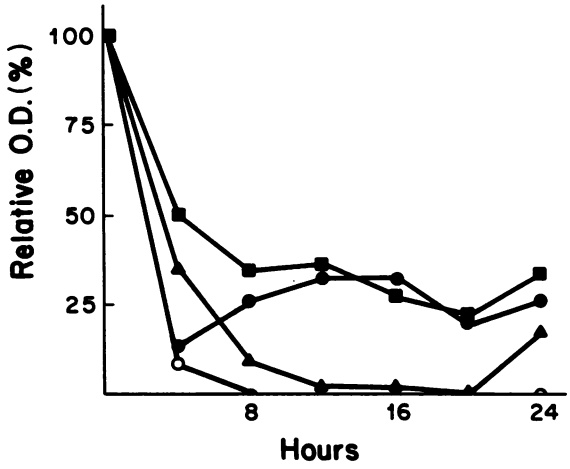

FIG. 4. Quantitation of immunochemical data from Fig. 3. Nuclear fractions of CHO cells stressed with different grp inducers were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and monitored by Western blotting with a rabbit anti-topoisomerase II antibody. The blots were photographed, and the negatives were scanned by using a densitometer. The optical density (O.D.) of the topoisomerase II band is normalized to that obtained with untreated cells and is plotted against the time under stress. The applied stresses were glucose deprivation (○), anoxia (▲), 10 mM 2-deoxyglucose (●), or 10 mM glucosamine (■).

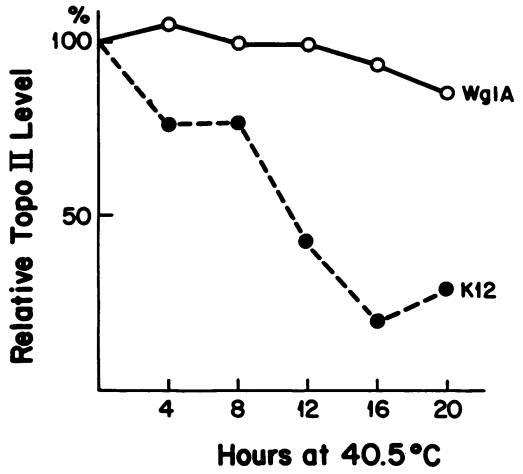

FIG. 5. Topoisomerase II (Topo II) levels in temperature-sensitive K-12 cells at the nonpermissive temperature. Wg1A cells (○) and K12 cells (●) were incubated at 40.5°C for the times indicated and then fractionated. The nuclear fractions were analyzed by Western blotting, and the level of topoisomerase II was determined and plotted relative to the control level for each line as described in the legend to Fig. 4. The control levels of topoisomerase II did not substantially differ between the parental and mutant cell lines.

when the same nitrocellulose filters were used, there was no change in 18S rRNA content following stress. These data argue that the rapid initial loss of the enzyme is more likely to be due to altered translation, protein degradation, or other processes than to diminished transcription.

DNA synthesis and cell cycle analysis. To understand the effect of the above alterations on DNA synthesis and cell cycle parameters, we examined the effect of glucose-regulated stresses on these endpoints. The effect of glucose-regulated stresses on DNA synthesis is presented in Fig. 7, in which the effects of 2-deoxyglucose and glucosamine on [^{3}H]thymidine incorporation are coplotted with the percentage of cells in the S phase. It is evident that a decline in DNA synthesis was obtained upon exposure to a glucose-regulated stress in coincidence with a decline in the percentage of cells in the S phase. These changes, however, appeared to lag behind the decline in topoisomerase II levels (i.e., an almost complete effect on topoisomerase II was obtained within the first 4 h [Fig. 3], whereas the majority of the decline in DNA synthesis occurred later than 4 h [Fig. 7]). This effect is also indicated by the data presented in Fig. 8, showing that the increase in the number of cells with a G_1 DNA content was most pronounced after 5 h of exposure to 2-deoxyglucose (10 mM), glucosamine (10 mM), or the calcium ionophore A23187 (10 μM). Similar results were obtained with glucose deprivation and exposure to anoxia.

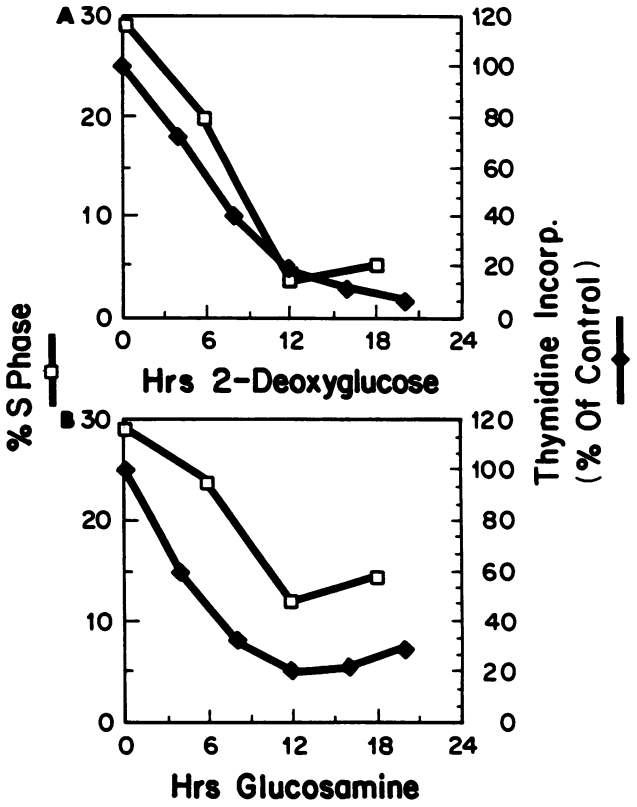

FIG. 7. Effect of glucose-regulated stresses on DNA synthesis and the percentage of S-phase cells. CHO cells were stressed with 10 mM 2-deoxyglucose (A) or 10 mM glucosamine (B) for the times indicated. Coplotted are the percentage of cells in the S phase (□, expressed as percentage of the control) and the percentage of [^{3}H]thymidine incorporation (◆, also presented as percentage of control). A concomitant decline in both indicies is indicated.

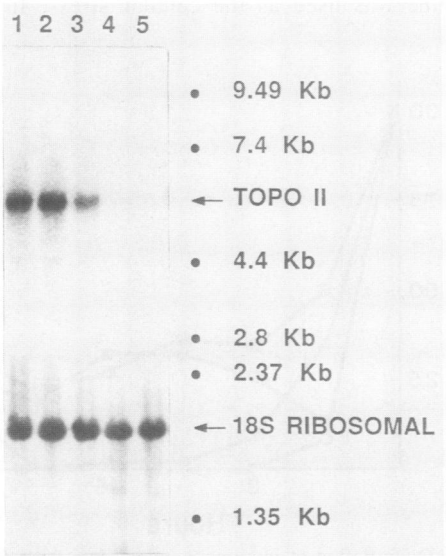

FIG. 6. Topoisomerase II (TOPO II) mRNA levels after exposure to 2-deoxyglucose. Exponentially growing CHO cells were stressed with 10 mM 2-deoxyglucose for 6 h (lane 2), 12 h (lane 3), 18 h (lane 4), and 24 h (lane 5) or 0 h (control, lane 1). Cellular RNAs were extracted, electrophoresed, transferred to nitrocellulose paper, and probed with nick-translated human topoisomerase II cDNA. The nitrocellulose paper was then further probed with a nick-translated 18S ribosomal cDNA. kb, Kilobases.

Effect of grp stress on DNA strand breakage. The cytotoxicity of etoposide is thought to be due to DNA damage secondary to alterations in topoisomerase II obtained in the presence of the drug, and, in general, etoposide cytotoxicity correlates well with DNA strand scission. Thus, it was of interest to correlate the induction of cellular resistance and the loss of enzyme content with etoposide-induced DNA breakage. Chinese hamster ovary cells were exposed to either 10 or 50 μM etoposide for 1 h following 0, 6, 12, 18, or 24 h of exposure to 10 mM 2-deoxyglucose. Since the data for 10 and 50 μM etoposide are virtually identical when

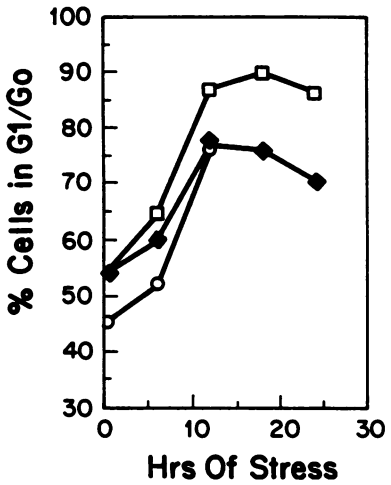

FIG. 8. Dependence of the number of cells in the G_1 phase on grp stress. CHO cells were treated with 10 mM 2-deoxyglucose (○), 10 mM glucosamine (◆), or 10 μM A23187 (□) for the times indicated, and the percentage of cells with a G_1 DNA content was plotted as a percentage of the control. The principal change was obtained at times in excess of 5 h.

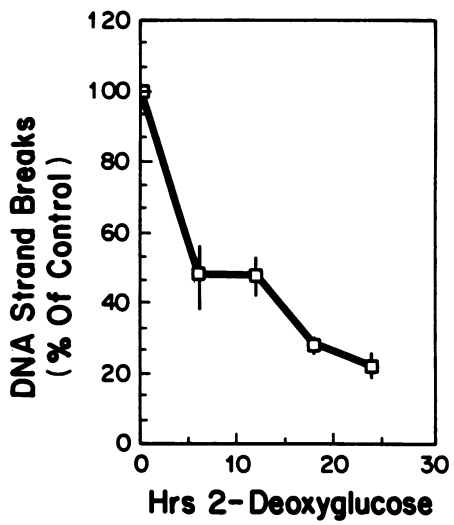

FIG. 9. Effect of 2-deoxyglucose stress on DNA strand breakage. [^{14}C]thymidine-labeled CHO cells were stressed with 2-deoxyglucose for various times. Cells were later treated with 10 or 50 μM etoposide for 1 h as described in Materials and Methods and analyzed by alkaline elution. The DNA break frequency was presented as a percentage of that of the control.

expressed as a percentage of control values, they have been pooled in Fig. 9 for the purposes of representation. A rapid loss of drug effect was observed during the first 6 h of stress, after which a more gradual reduction in DNA breakage occurred. The time course of these data correlates well with the time course of increase in resistance (Fig. 1) and the loss of enzyme content (Fig. 4).

DISCUSSION

Resistance to the drug adriamycin can be obtained by exposing cells to anoxia and other stresses which specifically lead to the induction of a set of stress proteins, of 76, 97, and 170 kDa, known as grps (33). This glucose-regulated inducible resistance appears to differ from the well-studied pleiotropic multidrug resistance (32) on the basis of the lack of correlation of cross-resistant drugs (C. S. Hughes, J. W. Shen, and J. R. Subjeck, Cancer Res., in press), its transient nature, and its independence from cellular drug retention (33, 51). One inducer of this stress response is exposure to an anoxic environment (31, 39), and Teicher et al. (40) have shown that anoxia also leads to resistance to the drug etoposide. The present study demonstrates that a plethora of glucose-regulated stresses leads to substantial resistance to etoposide in CHO cells. In addition, K12 cells, which are temperature sensitive for the induction of grps (15, 18), express resistance to etoposide in congruence with the expression of this set of stress proteins at the nonpermissive temperature. Etoposide resistance is not expressed when parental Wg1A cells are incubated at the same temperature. Furthermore, this cellular resistance is detectable at the level of DNA strand scission, as indicated from studies with 2-deoxyglucose as the stress.

In light of these observations and the relationship recognized to exist between nuclear topoisomerase II content and resistance to etoposide, this study was extended by examining topoisomerase II levels in stressed cells. In CHO cells exposed to glucose-regulated stresses and in the grp-induced K12 temperature-sensitive mutant cell line incubated at the nonpermissive temperature, expression of etoposide resistance is accompanied by a reduction in the amount of topoisomerase II. In the K12 cells, the depletion of topoisomerase II is related to the temperature-sensitive defect, since levels of the enzyme are not substantially altered in parental Wg1A cells incubated at the same temperature, again in agreement with the lack of drug resistance observed in the parental cells. These studies collectively argue that the depletion of topoisomerase II represents a consistent characteristic of the glucose-regulated stress response. Since sensitivity to etoposide requires the presence of topoisomerase II, this aspect of the glucose-regulated state would appear to be responsible, in part, for the glucose-regulated mode of drug resistance.

The K12 temperature-sensitive mutant cell line was initially isolated as a cell cycle mutant arrested in G_1 at the nonpermissive temperature (41) and was later recognized to express specific proteins identified as grps (15, 18). Furthermore, quiescense has been generally associated with a decline in the level of the topoisomerase II enzyme (38). In the present study, cell cycle analysis indicates that the application of a glucose-regulated stress produces a redistribution of an asynchronous population of log-phase CHO cells into a G_1/G_0 state, with a corresponding decline of tritiated thymidine incorporation into DNA. However, the present study also indicates that although the movement of cells into G_1/G_0 and the decline in DNA synthesis obtained are approximately coincident events, both changes lag behind the depletion of topoisomerase II obtained in the stressed cells. Since the topoisomerase II level is considered to increase in G_2 (6, 12), the "reciprocal" appearance of Fig. 4 and 8 suggests that the reductions in topoisomerase II levels observed are not secondary to the movement of stressed cells into a quiescent state (i.e., the principal effect in Fig. 4 is obtained in less than 4 h, whereas that in Fig. 8 is seen between 5 and 10 h).

Another pathway recognized to diminish the topoisomerase II content occurs through an inhibition of cellular protein synthesis (6). This observation does not appear to be applicable to the phenomenon described in this report, since

many of the conditions examined here do not reduce cellular protein synthesis. Therefore, topoisomerase II appears to be a uniquely stress-sensitive nuclear protein in CHO cells, being rapidly depleted during a glucose-regulated stress. In analogy to the studies described here, it has been long recognized that severe heat shock leads to adriamycin resistance (11). Recent studies suggest that this observation may be accounted for by a reduction in intracellular drug levels in the heated cells (23), an effect not obtained during exposure to a glucose-regulated stress (33). In addition, thermotolerance-inducing 5- or 10-min 45°C heat shocks substantially increase topoisomerase II levels in CHO cells during periods when cells express resistance to etoposide and adriamycin and at times corresponding to the initial expression of thermotolerance (J. Subjeck and J.-W. Shen, manuscript in preparation).

Although a substantial decline in topoisomerase II content occurs during a glucose-regulated stress, the relationship of this stress response to the reduction in the level of this enzyme is unclear. Other studies have indicated that topoisomerase II can be phosphorylated (2, 46) or ADP ribosylated (8). Studies with the K12 cell line suggest that a temperature-sensitive defect in glycosylation is, in some manner, capable of activating the promoter of the major glucose-regulated gene encoding the 76-kDa grp (17), and inhibition of glycosylation is considered to be a common characteristic of many stresses leading to the induction of the grps. It is not presently understood how alterations in glycosylation and topoisomerase II levels might be related, although recent studies have described novel O-linked *N*-acetylglucosamine modifications of nonluminal nuclear pore complex proteins (13, 34). It is of interest that the depletion of topoisomerase II observed in this study is a selective phenomenon among other detectable nuclear proteins, suggesting some degree of specificity. In addition, the decline in protein levels is not a result of transcriptional regulation, as indicated by the fact that mRNA levels are not altered during the period in which the protein disappears. These data suggest a novel pathway for the regulation of topoisomerase II which has not been described previously. Finally, this decline in topoisomerase II content is not consistently reflected by a depletion in cellular ATP levels, since ATP levels are not altered during exposure to glucosamine (Locke and Rass, unpublished data).

The data presented in this study suggest that the mechanism by which glucose-regulated, stressed cells become drug resistant is through the depletion of topoisomerase II, although other mechanisms may also contribute. It has been suggested that the poor response to chemotherapy of well-differentiated, slow-growing tumors might be accounted for by reduced levels of topoisomerase II and, conversely, that high levels of enzyme in rapidly growing tumors might render them sensitive to topoisomerase II-targeted therapy (21). Anoxia and/or glucose deprivation can occur within tumors during growth and as a function of irregular or poor vascularization (10, 30, 47). That cells in a tumor can exist in a grp-induced state is supported by studies which show that increased levels of the 76-kDa grp are observed in larger, partially necrotic, RIF-1 tumors (33; Shen, unpublished data). Thus, even within a (rapidly) proliferating tumor, a significant resistance to topoisomerase II-targeted drugs may occur as a result of passage of tumor regions into a glucose-regulated state. Interestingly, the data presented here also suggest that tumor cells in the corresponding tumor regions would exist in a growth-arrested, quiescent state.

In conclusion, this study describes the effect of a specific category of stresses on certain cellular parameters associated with proliferation, the significance of which is twofold. First, several previous studies describe changes in protein glycosylation obtained during glucose-regulated stress. However, the pathway by which such changes in the endoplasmic reticulum are transmitted to the nucleus, leading to the expression of glucose-regulated genes, is unknown. Indeed, it is not known whether the repertoire of glucose-regulated stresses consistently affects any other aspect of nuclear structure or activity. The present study indicates that these glucose-regulated stresses lead to timely and substantial changes in nuclear composition and function. Whether any of these nuclear changes lie in the pathway leading to the expression of stress proteins and/or represent a parallel but potentially interconnected aspect of this stress response remains to be determined. Interestingly, recent data indicate that although topoisomerase II is lost from isolated nuclei, in some cases (at least), no significant change in protein level is observed is total-cell extracts (Shen and Subjeck, unpublished data). This would suggest that this observation is not related to protein synthesis and/or degradation but, rather, to the binding of the protein in the nuclear fraction or to nuclear transport. We are presently examining these possibilities. Second, since anoxia and glucose starvation are physiologically relevant states, these data also define a pathway via which cells can become resistant to an important category of antineoplastic agents in tumors in situ. The underlying molecular basis responsible for the loss of topoisomerase II during a glucose-regulated stress response and the possible relationship of this change as a potential prerequisite for the expression of stress genes are being examined.

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