Endoplasmic reticulum (ER) stress is associated with misfolding of ER proteins and triggers the unfolded protein response (UPR). The UPR, in turn, helps restore normal ER function. Since fastidious N-linked glycosylation is critical for folding of most ER proteins, this study examined whether metabolic interconversions of precursors used for glycan assembly were controlled by the UPR. Thus, eight enzymes and factors with key roles in hexose phosphate metabolism were assayed in cytoplasmic extracts from primary dermal fibroblasts treated with UPR inducers. Stimulation of only one activity by the UPR was detected, AMP-independent glycosylphosphatidylinositol (GPI). GP activation required only 20 min of ER stress, with concurrent decreases in cellular glycogen and elevations of its metabolites Glc-1-P and Glc-6-P. Addition of phosphatase inhibitors to enzyme extracts from unstressed cells mimicked the effect of ER stress on GP activity, suggesting that phosphorylation of GP or a regulatory factor was involved. These data show that the UPR can modulate hexose metabolism in a manner beneficial for protein glycosylation. Since activation of GP appears to occur by a rapid post-translational process, it may be part of a general strategy of ER damage control, preceding the well-known transcription-dependent processes of the UPR that are manifested hours after the occurrence of ER stress.

Endoplasmic reticulum (ER) stress initiates signals that emerge from the ER lumen, and activate cytoplasmic and/or nuclear responses, which in turn alter ER function. The general paradigm of ER stress signaling is termed the unfolded protein response (UPR), which can be triggered by agents that cause misfolded proteins to accumulate within the ER lumen, such as dithiothreitol (DTT), tunicamycin (TN), thapsigargin, castanospermine (CSN), and azetidine-2-carboxylic acid (AZC) (4). Typically, ER stress activates the stress sensors Ire1p and ATF6 (3) to cause transcription of genes encoding chaperones, folding enzymes, and other proteins that enhance ER function. Appearance of the respective gene products occurs several hours after initiation of ER stress. For such ER proteins, there is no evidence that regulation by the UPR involves post-translational import or activation. Additionally, ER stress can temporarily inhibit cellular protein synthesis, lessening the load of misfolded protein entering the lumenal space. This occurs within 10–20 min of the application of ER stress by rapid post-translational phosphorylation of eIF2α by the PKR-like ER kinase or PERK (5). Translation arrest is reversible (for example, lasting 2 h in fibroblasts, Ref. 4), allowing the subsequent translation of mRNAs encoded by UPR-responsive genes.

Previous studies from this laboratory with primary dermal fibroblasts identified another rapid effect of the UPR, involving N-linked glycosylation of ER proteins (4, 6). ER quality control is highly dependent upon covalent attachment of the oligosaccharide Glc₃Man₉GlcNAc₂ to specific asparaginyl residues of nascent ER proteins. This requires the synthesis of a lipid-linked oligosaccharide (LLO), Glc₃Man₉GlcNAc₂-P-P-dolichol. TN is a specific inhibitor of the synthesis of this LLO and, as a result, its application with cells causes ER protein misfolding and ER stress. Conditions that cause accumulation of premature LLO intermediates also result in ER stress (7). However, it was found that the UPR can compensate by promoting extension of such premature LLOs to Glc₃Man₉GlcNAc₂-P-P-dolichol (6). Of particular interest is DTT-induced stress, which stimulates LLO synthesis within 20 min, and is attenuated by adaptation of cells to stress (4). This suggests a form of stress relief in which one or more components of the pre-existing pathway for generation of Glc₃Man₉GlcNAc₂-P-P-dolichol is activated post-translationally, rather than being synthesized de novo. No evidence was obtained for UPR activation of hexose transport or transferases involved in synthesis of Glc₃Man₉GlcNAc₂-P-P-dolichol (6). Since multiple mannolosylated intermediates (Man₉₋₅GlcNAc₂-P-P-dolichol) were all extended by the UPR, it is feasible that the UPR acts by increasing synthesis of one or more precursors of the mannosyl residues.

In this study we present evidence that AMP-independent glycogen phosphorylase (GP) can mediate enhanced LLO extension by the UPR. Activation of GP is rapid, and may be part of a general program of ER damage control that precedes transcriptional events regulated by the UPR.

EXPERIMENTAL PROCEDURES

Cell Culture and Related Procedures—Human dermal fibroblasts, obtained from various sources (see below, Table II), were cultured and subjected to ER stress with DTT, TN, CSN, or AZC as described (6). Cells were cultured continuously in medium containing 5 mM glucose in all experiments presented. To verify the effects of UPR inducing treatments (except TN) on LLO synthesis, controls (not shown) were performed with cells labeled with 40 μCi/ml [2-3H]mannose, in which case a 20-min incubation with medium containing 0.5 mM glucose was used. [3H]LLOs were extracted with chloroform/methanol/water (10:10:3),...
and ["H]oligosaccharides were released from dolichol-P-P and analyzed by HPLC as described (6).

Cytoplasmic Enzyme Extracts—Cell monolayers were treated with streptolysin-O (SLO (Murex brand, distributed by Corgenix, UK)) to selectively permeabilize the plasma membranes as described (8, 9), allowing collection of diffusible cytoplasmic components with minimal physical perturbation or organelle breakage. Each 100-mm dish contained ~1 × 10^6 cells. After treatment with SLO on ice for 4 min, unbound SLO was removed by washing the monolayers with ice-cold phosphate-buffered saline. 2 ml of modified transport buffer (containing 78 mM KCl, 4 mM MgCl_2, and 50 mM Na-HEPES, pH 7.4, but lacking DTT as originally formulated, Ref. 8) prewarmed to 37 °C were added. After 15 min at 37 degrees, the dishes were placed on ice for an additional 5 min. The buffer was collected and used for measurements of hexose-metabolizing enzymes. Protein was measured with a dye binding assay (Bio-Rad) with bovine serum albumin as a standard. Comparisons of extracts made this way with extracts made with 1% Triton X-100 showed that Triton X-100 recovered 5–6 times as much protein, but a similar amount of total GP (not shown). The various stress treatments described in this study did not influence the amount of total protein recovered in cytoplasmic extracts.

Assays for Hexose Phosphate-metabolizing Enzymes—Assays were linear over the incubation periods indicated. Except for PFK, all enzymes in 0.2 ml of cytoplasmic extract were assayed in 1 ml of buffer containing 50 mM K-HEPES, pH 7.2, 25 mM KCl, and 5 mM MgCl_2. Reactions were performed in the presence of 1.5 units of glucose-6-P dehydrogenase (Sigma, catalog number G5829) and 0.2 mM NADP^+ (Sigma, catalog number N0505), and product was assessed spectrophotometrically at 340 nm for the reduction of NADP^+ to NADPH, as described (10). Except for PFK, the incubation times at 37 °C, and other reaction components, are indicated below for each enzyme. In all cases, assay values with blank reactions lacking the enzyme substrate were subtracted out. All data were normalized to protein content.

Hexokinase: reactions were incubated for 15 min with 1 mM t-glucose and 1 mM Mg-ATP. Phosphoglucoisomerase (PGI): reactions were incubated for 15 min with 1 mM fructose 6-phosphate. Phosphomannose isomerase (PMI): reactions were incubated for 2 h with 0.5 mM mannose 6-phosphate and 7.13 units PGI (Sigma, catalog number P5381). Phosphomannomutase (PMM): reactions were incubated for 2 h with 0.5 mM mannose 1-phosphate, 0.1 mM glucose 1,6-bisphosphate, 0.35 units PMI (Sigma P5153), and 7.13 units of PGI. Fructose-1,6-bisphosphatase: reactions were incubated for 15 min with 10 mM fructose 1,6-bisphosphate and 7.13 units of PGI. Phosphofructokinase (PFK): 0.2 ml of extract was mixed with 1 ml of buffer containing 1 mM fructose 6-phosphate, 0 or 1 mM Mg-ATP, 50 mM Tris-Cl, pH 8.0, 0.1 mM Na_2EDTA, 6 mM MgCl_2, 0.16 mM NADH, 0.4 units of aldolase (Roche Diagnostics cat. 109762), and 0.4 units of a-glycerophosphate dehydrogenase (Roche Diagnostics cat. 127752), and incubated for 15 min at 37 °C. The ATP-dependent formation of fructose 1,6-bisphosphate was determined with an enzyme-linked method (11).

Assay for Cytoplasmic Activators of Phosphofructokinase and Preparation of Alkaline Extracts—Fructose 2,6-bisphosphate (or similar activators) was measured by activation of PFK as described (12, 13). Rabbit muscle PFK (Sigma cat. F2129) and PFK in extracts from control and stressed cells were tested. Activators were obtained by alkaline extraction as follows. 100-mm dishes were placed on ice and washed with ice-cold phosphate-buffered saline twice. 1 ml of 0.1 M NaOH (ice-cold) was added, and cells were scraped, transferred to a glass tube, and sonicated for 20 s. The mixture was heated at 80 degrees for 5 min. Samples were cooled on ice and centrifuged for 20 min at 14,000 rpm in a microcentrifuge. The supernatants were neutralized using 1 M HEPES (free acid) and added to PFK assays. In our hands, PFK was activated 2–3-fold by extracts from control cells.

Measurement of Glycerogen Phosphorylase (GP)—Extracts prepared by SLO permeabilization (see above) were assayed for GP activity (14). A 0.2-ml extract was diluted into 1 ml of assay buffer containing 20 mM sodium phosphate (pH 7.2), 2 mM MgSO_4, 1–2 mM NADP^+, 2 μg/ml glucose-6-phosphate dehydrogenase, 3 units/ml rabbit muscle PGM, and 3 μg/ml glucose 1,6-bisphosphate. When indicated, 5 mM 5′-AMP was included. The reaction was initiated by adding 100 μl of 10 mg/ml glycogen (Sigma cat. G1508). GP activity was determined spectrophotometrically at 340 nm over a period of 5 h at 37 °C, and values for blank reactions lacking extract were routinely subtracted out. Over this period assays were linear for both time and amount of extract. The reaction was strictly dependent upon the presence of glycogen, PGM, and orthophosphate (not shown). Data presented for GP activity in the presence of AMP represent the total activity measured, without subtraction of activity obtained in the absence of AMP.

Measurement of Glucose 6-Phosphate by Enzyme Assay—Cellular alkaline extracts containing hexose phosphates (see above) were used. Since some hexose phosphates can be modified by alkali, key results were corroborated (not shown) with 70% ethanol extracts. Assays (1 ml) included 50 mM Tris-Cl (pH 7.5), 10 mM MgCl_2, 0.25 mM NADP^+ and 0.2 μl of extract. 2 μg/ml glucose-6-phosphate dehydrogenase (Sigma G7877) was added, and absorbance at 340 nm was measured after 15 min. Controls performed in the absence of glucose-6-phosphate dehydrogenase were subtracted out.

Measurement of Hexose Phosphates by Fluorophore-assisted Carbohydrate Electrophoresis (FACE)—Cell monolayers were extracted by scraping into 70% ethanol. The extract was clarified by centrifugation, dried under N_2(g), dissolved in water, and applied to a 1-ml column of Dowex AG1-X2 (formate form). After washing with 20 ml of water, which removed essentially all neutral hexose, hexose phosphates were eluted with 15 ml of 4 M formic acid (15) and dried under N_2(g). Recovery of a Glc-6-P standard was greater than 90% (not shown). However, hexose 1-phosphates were highly sensitive to hydrolysis, and under these conditions more than 90% of Glc-1-P standard was recovered as glucose. Thus, glucose appearing in the 4 M formic acid eluate represented Glc-1-P. Hexoses and hexose phosphates were modified with 2-aminoacridone (AMAC) and separated with a modified monosaccharide composition gel (16) composed of a 20% acrylamide separating gel, containing 50 mM K-HEPES, pH 7.2, 2 mM MgSO_4, 1 ml glucose-6-phosphate dehydrogenase (Sigma G7877) was added, and absorbance at 340 nm was measured after 15 min. Controls performed in the absence of glucose-6-phosphate dehydrogenase were subtracted out.

Measurement of Hexose Phosphates by Fluorophore-assisted Carbohydrate Electrophoresis (FACE)—Cell monolayers were extracted by scraping into 70% ethanol. The extract was clarified by centrifugation, dried under N_2(g), dissolved in water, and applied to a 1-ml column of Dowex AG1-X2 (formate form). After washing with 20 ml of water, which removed essentially all neutral hexose, hexose phosphates were eluted with 15 ml of 4 M formic acid (15) and dried under N_2(g). Recovery of a Glc-6-P standard was greater than 90% (not shown). However, hexose 1-phosphates were highly sensitive to hydrolysis, and under these conditions more than 90% of Glc-1-P standard was recovered as glucose. Thus, glucose appearing in the 4 M formic acid eluate represented Glc-1-P. Hexoses and hexose phosphates were modified with 2-aminoacridone (AMAC) and separated with a modified monosaccharide composition gel (16) composed of a 20% acrylamide separating gel, containing 50 mM K-HEPES, pH 7.2, 2 mM MgSO_4, 1 ml glucose-6-phosphate dehydrogenase (Sigma G7877) was added, and absorbance at 340 nm was measured after 15 min. Controls performed in the absence of glucose-6-phosphate dehydrogenase were subtracted out.

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and F1,6BPase activities were all measured in enzyme-linked assays and are reported as \( \mu \text{mol of NADPH formed per min.} \) PFK is measured as \( \mu \text{mol of Fru-1,6-bisP formed per min.} \) Fru-2,6-BP-like activity per \( 10^6 \) cells is reported as the equivalent pmol of authentic Fru-2,6-BP giving the same enhancement of PFK activity. Parentheses indicate the number of times each stress treatment was tested.

Measurement of Glycogen—Glycogen was extracted from cell monolayers in 100-mm dishes, pre-chilled on ice, and washed with ice-cold PBS, by scraping with 1 ml of ice-cold 70% perchloric acid followed by sonication at 0 degrees (17). After removal of insoluble material by centrifugation, aliquots were used for determination of glucose with or without prior enzymatic degradation of glycogen. Glycogen degradation was achieved by combining 0.4 ml of extract with 0.2 ml of 1 M KHCO3 –7.3, and centrifuged at 4000 rpm for 15 min. Glucose was assayed by mixing up to 0.1 ml of the supernatant with 1 ml of buffer containing 0.3 M triethanolamine chloride, pH 7.5, 1 mM ATP, 0.1 mM NADP+ by mixing up to 0.1 ml of the supernatant with 1 ml of buffer containing the same enhancement of PFK activity. Parentheses indicate the number of times each stress treatment was tested.

Measurement of Glycogen—Glycogen was extracted from cell monolayers in 100-mm dishes, pre-chilled on ice, and washed with ice-cold PBS, by scraping with 1 ml of ice-cold 70% perchloric acid followed by sonication at 0 degrees (17). After removal of insoluble material by centrifugation, aliquots were used for determination of glucose with or without prior enzymatic degradation of glycogen. Glycogen degradation was achieved by combining 0.4 ml of extract with 0.2 ml of 1 M KHCO3 and 2 ml of 1 mg/ml amyloglucosidase (Sigma A3514) solution, followed by shaking at 40 °C for 2 h. The incubation was stopped by adding 1 ml of 70% perchloric acid, neutralized with 2–3 mg solid KHCO3, to a pH of 6.9–7.3, and centrifuged at 4000 rpm for 15 min. Glucose was assayed by mixing up to 0.1 ml of the supernatant with 1 ml of buffer containing 0.3 M triethanolamine chloride, pH 7.5, 1 mM ATP, 0.1 mM NADP+, 4 mM MgSO4, and 5 \( \mu \text{g/ml glucose-6-phosphate dehydrogenase.} \) After incubation at 37 °C for 5 min, absorbance at 340 nm was determined. Hexokinase (Sigma H5625) was added to a final concentration of 1.4 units/ml. After an additional 5 min at 37 °C, absorbance at 340 nm was again determined, from which the first reading was subtracted to determine the glucose-specific change in absorbance. After subtraction of values obtained without amyloglucosidase treatment and comparison with standard glucose samples, the quantity of glycogen-derived glucose was determined.

**RESULTS**

**Many Activities Associated with Hexose Metabolism Are Unaffected by ER Stress**—Compared with tissues such as liver, brain, and muscle, little is known about regulation of glycolysis, gluconeogenesis, or glycogenolysis in dermal fibroblasts. A number of regulatory points in hexose metabolism with the potential to modulate LLO mannosylation were therefore evaluated to determine whether they might be regulated by the UPR (Fig. 1). ER stress conditions shown previously to stimulate the glucose-specific change in absorbance. After subtraction of values obtained without amyloglucosidase treatment and comparison with standard glucose samples, the quantity of glycogen-derived glucose was determined.

**FIG. 2.** ER stress increases the AMP-independent activity of glycogen phosphorylase, but not the AMP-dependent activity, in cytoplasmic extracts of dermal fibroblast cultures. In this and Figs. 3–6, experiments with adult fibroblast CRL-1904 (ATCC) are shown. Extracts were prepared by permeabilization with SLO. GP activities were measured as described under “Experimental Procedures.” Panel **A**, the effects of varying 5’-AMP concentrations were tested. Panel **B**, the effects of varying concentrations of caffeine were tested in the absence or presence of 5 mM AMP. Panel **C**, fibroblasts were cultured in the absence or presence of 2 mM DTT for 20 min. \( n = 8 \) or 5 \( \mu \text{g/ml TN for 1 h.} \) and GP activities (mean ± S.E.) were measured in the absence (–AMP) or presence (+AMP) of 5’-AMP.
late LLO extension were chosen. These conditions did not cause cytoplasmic stress, itself a potential regulator of hexose metabolism.\(^2\) DTT was particularly useful because it acted within 20 min of addition to cells, and its effects on LLO extension were due to an ER stress response rather than a direct chemical reduction of a regulatory enzyme (4). However, for the current study two significant modifications were made. First, cells were maintained continuously in medium with 5 mM glucose. In prior studies, a 20-min period in 0.5 mM glucose medium was necessary to provide a pool of truncated LLOs. Second, TN could not be used in prior studies since it directly inhibits LLO

| Table II |
|----------|
| Effects of DTT- and TN-induced ER stress on 5'-AMP-dependent and-independent glycogen phosphorylase activities in primary dermal fibroblasts |

Glycogen phosphorylase was assayed without or with 5 mM 5'-AMP in cytoplasmic extracts from cells treated in the absence (control) or presence of 2 mM DTT for 15 min, or 5 μg/ml TN for 60 min, and normalized to protein content, as described under “Experimental Procedures.” Cultures with CRL designations were from the American Type Culture Collection. F-12 was a gift of Dr. H. Freeze, Burnham Institute, and F21–3 was obtained from National Psoriasis Foundation Tissue Bank. Adult donors were clinically normal. Pediatric donors were normal or had clinical abnormalities not expected to affect the parameters measured here. For reasons that are unclear, the average GP activities among the 5 adult cultures (–AMP, 0.072; +AMP, 0.21) were higher than those for four pediatric cultures (–AMP, 0.034; +AMP, 0.095). GP activity in only one of 9 cultures, pediatric CRL-1474, did not respond to ER stress. Since this culture also had the highest GP activity among the four pediatric cultures, there is a possibility of constitutive activation of GP.

Table III

| Fibroblast cultures | –AMP | +AMP |
|--------------------|------|------|
|                    | Control DTT TN | Control DTT TN |
| Adult CRL-1904     | 0.080 ± 0.005 204 ± 23 | 0.299 ± 0.015 110 ± 9 |
| (n = 8)            | (n = 6) (n = 4) | (n = 8) (n = 6) (n = 4) |
| CRL-1892           | 0.075 ± 0.003 188 ± 20 | 0.200 ± 0.011 113 ± 7 |
| (n = 7)            | (n = 5) | (n = 7) |
| CRL-1987           | 0.068 ± 0.007 170 | 0.192 ± 0.006 122 |
| (n = 2)            | (n = 1) | (n = 2) |
| F-12               | 0.066 ± 0.008 134 ± 6 | 0.170 ± 0.013 90 ± 4 |
| (n = 2)            | (n = 2) | (n = 2) |
| F21–3              | 0.068 ± 0.003 152 ± 8 | 0.178 ± 0.002 109 ± 3 |
| (n = 2)            | (n = 2) | (n = 2) |
| Pediatric CRL-7514 | 0.027 ± 0.0002 171 ± 16 | 0.085 ± 0.001 102 ± 4 |
| (n = 2)            | (n = 2) | (n = 2) |
| CRL-2114           | 0.026 ± 0.001 180 ± 71 | 0.068 ± 0.006 101 ± 6 |
| (n = 2)            | (n = 2) | (n = 2) |
| CRL-1513           | 0.037 ± 0.002 177 | 0.168 ± 0.004 97 ± 12 |
| (n = 2)            | (n = 2) | (n = 2) |
| CRL-1474           | 0.044 ± 0.001 102 | 0.120 ± 0.004 73 ± 6 |
| (n = 2)            | (n = 2) | (n = 2) |

\[^{a}\] CRL-7514: 4-year-old Caucasian male, neuroblastoma. CRL-2114: 2-year-old Black male, no clinical abnormalities recorded. CRL-1513: 4-year-old male, ethnicity unrecorded, multiple congenital defects (LLO profiles were normal and responsive to ER stress (4), inconsistent with CDG-I). CRL-1474: 4-year-old male, ethnicity unrecorded, glioma.

\[^{b}\] TN-treated CRL-1904 cultures had a set of controls (n = 6) different than those for DTT, with average activities of 0.082 ± 0.006 (–AMP) and 0.278 ± 0.006 (+AMP).

\[^{c}\] Not determined.

\[^{2}\] J. Shang and M. A. Lehrman, unpublished observations.

\[^{3}\] Glycogen phosphorylase was assayed without or with 5 mM 5'-AMP in cytoplasmic extracts from cells treated in the absence (control) or presence of 2 mM DTT for 15 min, or 5 μg/ml TN for 60 min, and normalized to protein content, as described under “Experimental Procedures.” Cultures with CRL designations were from the American Type Culture Collection. F-12 was a gift of Dr. H. Freeze, Burnham Institute, and F21–3 was obtained from National Psoriasis Foundation Tissue Bank. Adult donors were clinically normal. Pediatric donors were normal or had clinical abnormalities not expected to affect the parameters measured here. For reasons that are unclear, the average GP activities among the 5 adult cultures (–AMP, 0.072; +AMP, 0.21) were higher than those for four pediatric cultures (–AMP, 0.034; +AMP, 0.095). GP activity in only one of 9 cultures, pediatric CRL-1474, did not respond to ER stress. Since this culture also had the highest GP activity among the four pediatric cultures, there is a possibility of constitutive activation of GP.

\[^{4}\] For reasons that are unclear, the average GP activities among the 5 adult cultures (–AMP, 0.072; +AMP, 0.21) were higher than those for four pediatric cultures (–AMP, 0.034; +AMP, 0.095). GP activity in only one of 9 cultures, pediatric CRL-1474, did not respond to ER stress. Since this culture also had the highest GP activity among the four pediatric cultures, there is a possibility of constitutive activation of GP.

\[^{5}\] Activation of Glycogen Phosphorylase by ER Stress

Effects of DTT- and TN-induced ER stress on 5'-AMP-dependent and-independent glycogen phosphorylase activities in primary dermal fibroblasts

Glycogen phosphorylase was assayed without or with 5 mM 5'-AMP in cytoplasmic extracts from cells treated in the absence (control) or presence of 2 mM DTT for 15 min, or 5 μg/ml TN for 60 min, and normalized to protein content, as described under “Experimental Procedures.” Cultures with CRL designations were from the American Type Culture Collection. F-12 was a gift of Dr. H. Freeze, Burnham Institute, and F21–3 was obtained from National Psoriasis Foundation Tissue Bank. Adult donors were clinically normal. Pediatric donors were normal or had clinical abnormalities not expected to affect the parameters measured here. For reasons that are unclear, the average GP activities among the 5 adult cultures (–AMP, 0.072; +AMP, 0.21) were higher than those for four pediatric cultures (–AMP, 0.034; +AMP, 0.095). GP activity in only one of 9 cultures, pediatric CRL-1474, did not respond to ER stress. Since this culture also had the highest GP activity among the four pediatric cultures, there is a possibility of constitutive activation of GP.
Panel A, stresses were 2 mM DTT for 20 min. (S.E.) by enzyme assay as described under "Experimental Procedures." Panel A, stresses were 2 mM DTT for 20 min. (N = 8), 200 μg/ml CSN for 24 h (N = 2), or 5 μg/ml TN for 1 h (N = 4). Panel B, cells were treated with 2 mM DTT for periods up to 60 min. Panel C, cells were treated for 20 min with up to 2 mM DTT. In B and C, data are averages of duplicates.

FIG. 3. ER stress elevates cytoplasmic glucose-6-P. Fibroblasts were treated in the absence or presence of ER stress inducers, and cytoplasmic glucose-6-P was measured (nmols per 10^6 cells, mean ± S.E.) by enzyme assay as described for Fig. 3. Panel A, monosaccharide profiling gel showing 200 pmol of n-glucose 6-phosphate (lane 1), 200 pmol of n-galactose 6-phosphate (lane 2), 100 pmol of glucose 6-phosphate mixed with material from the 70% ethanol extract of 10^4 DTT-treated cells (lane 3), the sample used for lane 3 but without glucose 6-phosphate (lane 4), and the 70% ethanol extract of 2 × 10^6 unstressed cells (lane 5). The positions of the two standards, as well as AMAC-reacting material of unknown structure, are shown. Panel B, percentage increase of glucose 6-phosphate and galactose 6-phosphate due to ER stress, determined from the image in panel A. The 100% values per 10^6 cells were 0.9 nmol for glucose 6-phosphate and 11 nmol for galactose 6-phosphate.

AMP-independent Glycogen Phosphorylase Activity in Dermal Fibroblasts Is Increased by ER Stress—As shown in Fig. 2, panel A, GP activity was detectable in fibroblasts in both its more active (AMP-independent) and less active (AMP-dependent) forms. Although detailed information about this enzyme in fibroblasts is lacking, by extension from tissues such as liver and muscle it is likely that phosphorylation converts the less active form, GPa, to the more active form, GPh (18). Caffeine, which blocks AMP binding to GPh, inhibited the AMP-dependent activity but not the AMP-independent activity (Fig. 2, panel B).

When cells were treated with DTT, TN (Fig. 2, panel C), or AZC (not shown) at concentrations known to cause ER stress (4), AMP-independent GP activity in extracts increased, but AMP-dependent activity was unaffected. Activation of GP occurred within 20 min of DTT addition. Direct addition of DTT to assays, or its inclusion during extract preparation, did not affect GP activity (not shown). Thus, stimulation by DTT was not due to a direct chemical reduction of GP or a modulator of GP, as expected since the cytoplasmic environment in which GP exists is highly reducing. In addition to the fibroblast culture used in the experiments displayed in the figures, ER stress with DTT or TN increased GPa but not GPh activities in seven other adult and pediatric dermal fibroblast cultures (Table II). The stimulation of GP by ER stress did not exceed 2-fold, but the following sections provide evidence that this stimulation was significant.

Glycogenolysis Is Stimulated by ER Stress—Dermal fibroblasts contained, on average, 230 nmol of glycogen-derived glucose per 10^6 cells. In the presence of orthophosphate, GP converts glycogen into Glc-1-P. Small but consistent losses of glycogen were observed with DTT (23% of control, n = 13) and TN (12% ± 2%, n = 3). For DTT, the decrease occurred after 15 min of addition. Since cellular glycogen loss may be counterbalanced by its synthesis, the apparently weaker effect of TN may be related to the fact that it acted more slowly (within 1 h). Thus, the resulting glycogenolysis would be more easily compensated by glycogen synthesis. These losses of glycogen, though small, were more than adequate to account for downstream effects on hexose phosphates and LLOs (Table III).

ER Stress Elevates Glucosyl Phosphates—Fluorophore-assisted carbohydrate electrophoresis (FACE) measurements of Glc-1-P showed an increase of 30% above control after 15 min of DTT treatment and 60% after 30 min (data not shown). The net effect appeared substantial (Table III). Glc-1-P can be condensed with UTP by UDP-Glc pyrophosphorylase, generating UDP-Glc plus pyrophosphate. Alternatively, Glc-1-P can be converted to Glc-6-P by phosphoglucomutase. Thus, it was likely that a portion of any Glc-1-P generated would have been converted to these other metabolites. As shown with an enzyme-linked assay (Fig. 3), DTT, TN, and CSN-induced ER stresses consistently increased cytoplasmic Glc-6-P (panel A). CSN, a weak UPR inducer (4), gave the weakest response. The effect of DTT was observed within 5 min of addition to cell cultures (panel B), and was maximal after 15 min. Concentrations as small as 0.2 mM were effective (panel C). These results...
were in accord with the previous demonstration that LLO extension was strongly stimulated by 0.4 mM DTT and occurred within 20 min of addition to cultures (4).

DTT treatment also resulted in elevation of Glc-6-P when measured by FACE (Fig. 4, panel A). This increase (~5-fold, panel B) was greater than that measured by enzyme assay (~2-fold, Fig. 3). Yet, the net increases were similar, in the range of 3 to 4.5 nmol per 10^6 cells (Table III). This suggests that an interfering component in the extract may have contributed to the background in the enzymatic assay for Glc-6-P.

**Quantitative Assessment of Glycogenolysis as the Basis for LLO Extension**—Taken together these results indicate that ER stress activates GP, associated with glycogenolysis and generation of Glc-1-P and Glc-6-P. As shown in Table III, ER stress generates at least 100-fold greater equivalents of hexose-P than needed to support synthesis of Glc_3Man_9GlcNAc_2-P-P-dolichol in these cells as measured by FACE (16). Presumably, the bulk of the glycogen-derived hexose-P not used for LLO extension is available for glycolysis.

**Phosphatase Inhibitors Mimic the Effects of ER Stress on GP Activity**—In all systems previously examined, AMP-dependent GPb is converted to AMP-independent GPa by phosphorylation (18). Thus, a commercial mixture of phosphatase inhibitors was included during the preparation of extracts to optimize the determination of ER stress activation of GP. The results (Fig. 5) were unexpected. Phosphatase inhibitors had no effect on AMP-independent GP activity in extracts from stressed cells, but they increased the activity from unstressed cells to levels recovered from stressed cells. Thus, the inclusion of phosphatase inhibitors during extract preparation had the same effect on GP activity as treating intact cells with UPR inducers, but these were not additive. Presumably, the relevant phosphorylation event occurs not only in the cell, but also in the extract and/or under assay conditions. Otherwise, the inhibitor mixture should have been without effect. The implications of these data for regulation by phosphorylation are considered under the “Discussion” and in Fig. 6 below.

**DISCUSSION**

This study identified AMP-independent glycogen phosphorylase as a candidate for mediating the ER stress-dependent extension of LLO precursors and increase of Glc_3Man_9-

**FIG. 5. Phosphatase inhibitors mimic the effects of ER stress on GP activity.** A proprietary aqueous mixture of phosphatase inhibitors (Sigma cat. P5726) including sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole was used. Cells were treated with DTT or TN as in Fig. 3, and cytoplasmic extracts were prepared with or without 1% (v/v) inhibitor mixture added to the transport buffer. AMP-independent GP activity was measured as units/min/mg protein (mean ± S.E., n = 6 for DTT and n = 2 for TN).

**FIG. 6. Model for stimulation of LLO extension by UPR regulation of glycogenolysis.** Accumulation of LLO intermediates, such as by glucose depletion, results in ER stress. As shown in this study ER stress activates glycogen phosphorylase (GP). This, in turn, mobilizes hexose-P from glycogen, resulting in increased concentrations of precursor substrates needs for extension of LLO intermediates to mature LLOs. In this figure we speculate that the UPR may prevent dephosphorylation of GP to an AMP-dependent state. Other potential modes of regulation suggested by these data include production of a phosphatase inhibitor that protects GP from dephosphorylation, activation of a kinase that compensates for GP dephosphorylation, and/or phosphorylation of a separate regulator of GP.
GlcNAC$_2$-P-dolichol concentration. A number of trivial explanations for these changes had already been ruled out (Refs. 4 and 6; see the Introduction). Seven other candidate enzymes and factors were unchanged by ER stress. Experiments with phosphatase inhibitors were consistent with activation of AMP-independent GP activity by a phosphorylation-dependent mechanism. At present, it is not known whether ER stress controls one of the previously identified modulators of GP phosphorylation, such as phosphorylase kinase or protein phosphatase 1 or a novel phosphorylation-dependent modulator of GP. Although no change in GP activity was detected in assays in the presence of AMP, suggesting that the total amount of enzyme was unaffected, it remains possible that GP was also activated intracellularly by AMP or another cytoplasmic component that dissociated during analysis.

ER stress increased GP activity by no more than a factor of 2, and only small amounts of glycogen were consumed. However, activation of GP was significant. First, as summarized in Table III, the amount of hexose-P derived from glycogen is over 100 times the amount theoretically needed to produce substrates for LLO extension. Second, GP is activated, glucosylation is stimulated, and both Glc-1-P and Glc-6-P are produced by treatment times and concentrations of UPR inducers that are highly consistent with those that stimulate LLO extension (4). Third, both biochemical (6) and genetic (19) studies have shown that Glc-6-P is an important source of hexose for LLO synthesis. Fourth, in 3T3-L1 adipocytes, glycogen appears to act as a buffer against accumulation of LLO intermediates due to glucose deprivation (20). Formal proof that GP is the UPR effector responsible for extension of LLOs will require techniques that permit specific inhibition of GP activation.

The effects of GP activation by ER stress on the pathway leading from glycogen to UDP-Glc, which is the donor substrate for glucose-P-dolichol synthase, remain to be examined. However, two observations suggest that this is also enhanced. In cells that accumulate more Man$_{2-5}$GlcNAc$_2$-P-dolichol than Glc$_3$Man$_9$GlcNAc$_2$-P-dolichol due to glucose deprivation, ER stress increased the radiochemical amount of [3H]Glc$_3$Man$_9$GlcNAc$_2$-P-dolichol in cells when [3H]mannose was used to label mannosyl residues (6). Yet, the same stress treatments eliminated the appearance of [3H]Glc$_3$Man$_9$GlcNAc$_2$-P-dolichol when [3H]galactose was used to label glucosyl residues (data not shown). The latter process requires intracellular conversion of galactose to Gal-1-P, UDP-Gal, UDP-Glc, and glucose-P-dolichol. Since UDP-Glc is also formed from Glc-1-P resulting from glycogenolysis, these results could be explained if ER stress elevated UDP-Glc concentrations and diluted the UDP-[3H]glucose formed from [3H]galactose. In agreement with this idea, ER stress enhanced the glucosylation of LLOs in Congenital Disorder of Glycosylation Type-Ic cells, in which glucosylation of LLOs is deficient due to a mutation that reduces the activity of glucose-P-dolichol:Man$_9$GlcNAc$_2$-P-dolichol glucosyltransferase (4). Thus, elevation of UDP-Glc might stimulate glucose-P-dolichol production and help compensate for the enzymatic defect.

The rapid activation of GP, combined with the evidence for a role of phosphorylation, strongly suggests regulation by post-translational modification rather than transcription. Among the UPR signaling pathways that have been identified, this is most reminiscent of eIF2α phosphorylation by the ER stress sensing kinase PERK. In fibroblasts, both GP activation (this study) and eIF2α phosphorylation (4) occur within 15–20 min of application of ER stress. It will therefore be interesting to determine whether GP can be activated in a PERK-deficient cell, such as PERK (−/−) embryonic fibroblasts (21). Unlike transcriptional mechanisms, some post-translational modifications can be easily reversed. GADD34 has been identified as promoting dephosphorylation of eIF2α (22), so a similar mechanism may be responsible for deactivating GP. If ER stress generates a phosphatase inhibitor (Fig. 6), the UPR may control glycogenolysis by modulating cycling between phosphorylated and dephosphorylated GP.

Given these results and the information we have recently reported on the high priority of LLO extension in the UPR (4), the UPR appears to proceed in two phases. The first phase, which takes place within minutes of the generation of ER stress, may be responsible for ER damage control. This phase is characterized by temporary adjustments that can quickly counteract the causes of ER stress, such as by increasing the supply of hexose-P for LLO synthesis and energy production, and by decreasing the amount of protein entering the ER. However, long-term solutions would occur hours later during the second phase, characterized by remodeling of the ER in a manner requiring gene transcription.

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Note Added in Proof.—Corgenix no longer sells SLO. We found that Sigma product S-140 is also suitable.

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