Alterations in Lipid-linked Oligosaccharide Metabolism in Human Melanoma Cells Concomitant with Induction of Stress Proteins*

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The original goal of this study was to examine post-translational modifications of the stress proteins p32 and p34 in human and murine cells, respectively, following induction by sodium arsenite (13). In the course of preliminary experiments, we observed that arsenite challenge of these cultures resulted in increased incorporation of [3H]mannose into a component with a molecular mass on SDS-PAGE of 14 kDa (designated M14). p32 and p34 were not detectably mannosylated. We report an initial characterization of the induction of this molecule and its stability and turnover and tentatively identify the molecule as a lipid-linked oligosaccharide whose synthesis is significantly increased in cells exposed to a range of insults. To our knowledge, this is the first report documenting alterations in the metabolism of lipid-linked oligosaccharides under conditions that concomitantly induce stress protein synthesis.

Experimental Procedures

Cell Culture, Stress Conditions, and Metabolic Labeling—Human A375 melanoma cells were propagated in monolayer culture as described (14). Cells were seeded into 35-mm plastic dishes in 3 ml of DMEM and incubated overnight at 37°C. Confluent monolayers were rinsed three times with phosphate-buffered saline (GIBCO), exposed to stress agents in serum-free, low glucose DMEM (GIBCO) for 8 h, and radiolabeled with 200 μCi/ml [3H]mannose ([2-3H]mannose, 22 Ci/mmol, ICR Radiochemicals) or 10 μCi/ml [35S]methionine ([L-35S]methionine, >800 Ci/mmol, Amersham Corp.) for the last 4 h of stress. Confluent A375 cultures were heat-shocked by addition of prewarmed (43°C) DMEM supplemented with 2% fetal bovine serum. Sealed T-25 flasks were submerged in a 43°C water bath for 30 or 60 min. Following treatment, cultures were replenished for various times with medium prewarmed to 37°C and radioabeled during the last hour with 200 μCi/ml [3H]mannose in DMEM.

To study the kinetics of stress-induced alterations in glycosylation, cells were pulse-labeled with [3H]mannose for 1-20 h following the addition of sodium arsenite. The reversibility of stress-induced changes was investigated by challenging cells with arsenite for 8 h, followed by various recovery times in arsenite-free DMEM. During the last hour of each recovery, cultures were radiolabeled with [3H]mannose. To analyze the metabolic turnover of M14, cells were radiolabeled with [3H]mannose in DMEM during the last hour of an 8-h arsenite stress and then incubated with fresh DMEM, with or without arsenite, up to 1 h. To study M14 synthesis, cells were stressed with arsenite for 8 h in the presence of actinomycin D (Sigma) (1 μg/ml), cycloheximide (Behring Diagnostics) (5 μg/ml), or tunicamycin A, (Boehringer Mannheim) (250 μg/ml). Cells were radiolabeled with [3H]mannose for the last 4 h of stress.

Gel Electrophoresis and Fluorography—Cellular extracts were analyzed as described previously (13). Briefly, cell monolayers were solubilized on ice for 20 min in 10 mM Tris buffer, pH 7.6, containing 1% Nonidet P-40, 0.1% SDS, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, and 2 mM phenylmethylsulfonyl fluoride. Following centrifugation in an Eppendorf microcentrifuge, supernatants were mixed with an equal volume of sample buffer and boiled for 3 min. Equal amounts of protein or trichloroacetic acid-insoluble radioactivity were analyzed on one-dimensional SDS-PAGE using the discontinuous buffer system of Laemmli (15) with a 4.5% acrylamide stacking gel and a 12.5% acrylamide resolving gel. Equilibrium two-dimensional SDS-PAGE

The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; GlcitolNAc, N-acetylglucosaminitol.

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was performed according to Bravo (16). Trichloroacetic acid-insoluble radioactivity was estimated as described (13). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Chemical Co.) with bovine serum albumin as a standard. Molecular weight and pl protein standards (Pharmacia F-L Biochemicals), respectively.

Radioactivity associated with electrophoretically separated M14 was estimated by extracting excised gel slices with Protosol (Du Pont-New England Nuclear) and incubated with endoglycosidase H (endo-β-N-acetylglucosaminidase H, Miles Laboratories, Inc.) for different times at 37 °C. Ovalbumin and fetus were used as standards. Digests were boiled for 3 min and analyzed by one-dimensional SDS-PAGE.

Solubility in Chloroform/Methanol/Water—This was performed according to a standard protocol (17) with certain modifications. Cultures were sonicated in 0.02 M Tris buffer, pH 7.4, containing 0.15 M NaCl and centrifuged in a Beckman TL-100 ultracentrifuge at 220,000 × g for 35 min at 4 °C. Supernatants were removed and analyzed by one-dimensional SDS-PAGE. The insoluble pellet was extracted three times with chloroform/methanol/water (3:2:1); the combined lower phases were pooled, washed three times with chloroform/methanol/water (3:48:47), and evaporated to dryness under N₂. The interphase and pellet were washed three times with water and then extracted three times with chloroform/methanol/water (10:10:3). Extracts were evaporated and analyzed by one-dimensional SDS-PAGE.

Mild Acid Hydrolysis—Conditions for acid hydrolysis were as described (18). Briefly, SDS-solubilized cell extracts were incubated with 0.02 M HCl at 95 °C for 30 min. Acids were removed by repeated dissolution in water and evaporation. The hydrolyzed samples were analyzed by one-dimensional SDS-PAGE.

Characterization of Labeled Oligosaccharides Released from M14 by Mild Acid Hydrolysis—Aliquots of M14 purified from both stressed and unstressed melanoma cells by electrophoresis from one-dimensional SDS-PAGE were subjected to mild acid hydrolysis in 25% isopropanol alcohol containing 0.02 M HCl at 95 °C for 30 min. After neutralization with 0.02 M NaOH, released oligosaccharides were analyzed by gel filtration on Bio-Gel P-6 (200-400 mesh, 1.0 × 110 cm), equilibrated, and eluted with 10 mM Tris-HCl, pH 6.8. Fractions were collected and analyzed for radioactivity.

RESULTS

Glycosylation Patterns in Human Melanoma Cells Challenged with Sodium Arsenite—Treatment of human A375 melanoma cells with sodium arsenite enhanced [³H]mannose incorporation into a low molecular weight component that migrated on one-dimensional SDS-PAGE with an apparent mass of 14 kDa (Fig. 1). This molecule (referred to as M14) was also present in unstressed cells. Detectable elevation of radioactivity into M14 was elicited by 24 μM sodium arsenite, whereas maximal stimulation occurred at higher concentrations (72-96 μM). Similar results were obtained when either equal amounts of radioactivity or total cellular protein were analyzed. Parallel analysis of A375 cultures treated under identical conditions but radiolabeled with either [³5S]methionine or [³H]-amino acids failed to reveal a concomitant increase of protein synthesis in this molecular weight range, but enhanced synthesis of the major stress proteins (p100, p90, p73/72, and p32 kDa) was readily observed (Fig. 1). [³5S] Methionine incorporation into trichloroacetic acid-insoluble cellular material was not significantly inhibited by sodium arsenite (data not shown). Similar results were obtained with human colon carcinoma cells (Culo 201 and HT-29) and fibroblasts (CCD-21Sk and CCD-330) (data not shown).

On two-dimensional SDS-PAGE, radiolabeled M14 migrated as a single acidic spot with a pl of approximately 4.5 (Fig. 2). Parallel analysis of [³5S]methionine- or [³H]asparagine-labeled material failed to identify a co-migrating protein (data not shown). M14 extracted from control or stressed cultures displayed similar electrophoretic properties (Fig. 2). In addition, M14 was metabolically labeled with [³¹P]orthophosphate as judged by identical migrations on two-dimensional SDS-PAGE (data not shown).

Kinetics of M14 Expression—Enhanced [³H]mannose incorporation into M14 was detectable 1 h following challenge of A375 cells with sodium arsenite (48 μM) and peaked after 6-8 h (Fig. 3A). To investigate whether elevated levels of M14 were sustained in stressed cultures following removal of insult, A375 cells were challenged with arsenite (48 μM) for 8 h and allowed to recover in arsenite-free medium for up to 24 h. During the last hour, cells were radiolabeled with [³H]mannose. Recovery of M14 to pre-stress levels required approximately 6 h (Fig. 3B).

The stability of M14 expression was examined by challenging cells with arsenite (48 μM) for 8 h and radiolabeling with [³H]mannose for the last hour. The cultures were then replenished with medium and incubated in the presence or absence of arsenite for up to 1 h. Under these conditions, the chase half-life for M14 was approximately 15 min (Fig. 4). A similar value was found for M14 expression in control, unstressed cultures (data not shown).

M14 Expression in Response to Different Insults—Challenge of A375 cells with heavy metals (zinc, copper, cadmium, and nickel), sulfhydryl-reactive reagents (iodoacetamide and auranofin), amino acid analogs (L-azetidine-2-carboxylic acid and L-canavanine), disulfiram (a copper-chelating agent), or the calcium ionophore A23187 induced significant incorporation of [³H]mannose into M14 (Table I). Treatment with hyperthermia gave equivocal results. Heat shock (43 °C for 30 or 60 min) significantly inhibited cellular uptake of [³H]mannose, but incorporation of radiolabel into M14 was less severely affected (Fig. 5). At 1 h post-recovery, M14 expression, as determined by densitometry, was decreased 62% compared to control; but by 4 h, it had increased 27% over control values. Recovery to pre-stress levels required approxi-
FIG. 2. Analysis of M14 by two-dimensional SDS-PAGE. A375 cultures were challenged with sodium arsenite (48 μM) for 8 h and radiolabeled with [3H]mannose for the last 4 h. Cell extracts containing equivalent amounts of trichloroacetic acid-insoluble radioactivity (20,000 cpm) were analyzed by two-dimensional SDS-PAGE and fluorography as described under “Experimental Procedures.” IEF, isoelectric focusing.

FIG. 3. Kinetics of M14 expression. A, induction of M14 expression. A375 cultures were stressed with sodium arsenite (48 μM) for the indicated times (1–20 h) and radiolabeled with [3H]mannose for the last hour of stress. Lane C, control. B, recovery of M14 expression. Cells were exposed to sodium arsenite (48 μM) for 8 h and then incubated in arsenite-free DMEM for periods up to 24 h. During the last hour of recovery, cultures were labeled with [3H]mannose in DMEM. Lane CO, control cells incubated for 8 h in DMEM; lane C24, control cells incubated for 24 h in DMEM. Cell extracts were analyzed as described for Fig. 1.

TABLE I

| Stress agents which induce increased M14 expression | Concentration |
|---------------------------------------------------|---------------|
| Human A375 melanoma cells were stressed with the indicated agents and analyzed by one-dimensional SDS-PAGE as outlined under “Experimental Procedures.” |               |
| **Insult**                                         | **M**         |
| Hyperthermia (30 and 60 min at 43 °C)              |               |
| Amino acid analog                                  |               |
| L-Azetidine-2-carboxylic acid                      | 5 x 10^{-3}   |
| L-Canavanine                                       | 10^{-2}       |
| Calcium ionophore                                  |               |
| A23187                                             | 10^{-6}       |
| Heavy metals and sulphydryl reagents               |               |
| Sodium arsenite                                    | 5 x 10^{-5}   |
| Zinc chloride                                      | 2.5 x 10^{-5} |
| Copper chloride                                    | 10^{-3}       |
| Cadmium chloride                                   | 10^{-4}       |
| Nickel chloride                                    | 10^{-4}       |
| Iodoacetamide                                      | 10^{-4}       |
| Auranofin                                          | 5 x 10^{-7}   |
| Copper-chelating agent                             |               |
| Disulfiram                                         | 2 x 10^{-4}   |
| Tunicamycin A2 (250 ng/ml), which does not inhibit protein synthesis (23), caused nearly complete inhibition of [3H]mannose incorporation into M14 and other mannosylated material (Fig. 6). |               |

Enzymatic Digestion of M14—SDS-PAGE analysis revealed
Stress-induced Alterations in Glycosylation

Recovery at 37°C (hr)

C 1 2 4 8

FIG. 5. Induction of M14 expression by hyperthermia. A375 melanoma cells were heat-shocked at 43°C for 30 min, allowed to recover at 37°C for the indicated times (1-8 h), and radiolabeled with [3H]mannose for the last hour of recovery. Cell extracts containing equivalent amounts of protein (50 µg) were analyzed by one-dimensional SDS-PAGE fluorography and described under “Experimental Procedures.”

FIG. 6. Expression of M14 in the presence of cycloheximide, actinomycin D, or tunicamycin. A375 cultures were stressed with sodium arsenite (48 µM) for 8 h in the presence (+) or absence (−) of 5 µg/ml cycloheximide, 1 µg/ml actinomycin D, or 250 ng/ml tunicamycin. Cells were radiolabeled with [3H]mannose for the last 4 h of stress, and equal amounts of protein were analyzed as described under “Experimental Procedures.”

FIG. 7. Selective extraction of M14 in chloroform/methanol/water (10:10:3). A375 cultures were stressed for 8 h with sodium arsenite (48 µM) and radiolabeled with [3H]mannose for the last 4 h. Cells were sonicated in Tris buffer and centrifuged. The insoluble pellet was sequentially extracted with a 3:2:1 mixture of chloroform/methanol/water (lane 3, 40,000 cpm), followed by extraction with a 10:1:3 solution of chloroform/methanol/water (lane 4, 100,000 cpm). The insoluble pellet remaining after organic extractions (lane 5, 40,000 cpm) and total cellular one-dimensional SDS lysates from control (lane 1, 50,000 cpm) and arsenite-stressed (lane 2, 50,000 cpm) cultures are all included for comparison. All samples were dissolved in one-dimensional SDS-PAGE sample buffer and analyzed as described under “Experimental Procedures.”

that M14 isolated by electroelution from stressed A375 cells was partially degraded within 2 h by endoglycosidase H, an enzyme that hydrolyzes the di-N-acetylcjithobiose linkage of high mannose oligosaccharides which are N-linked to proteins or joined through a pyrophosphate bridge to dolichol carrier (19). Complete digestion required 24 h. In contrast, M14 was stable to prolonged (5 days) Pronase digestion and was not a substrate for peptide:N-glycosidase F. Control experiments indicated that the three enzymes were active against appropriate substrates (data not shown).

Solubility of M14 in Chloroform/Methanol/Water—Large lipid-linked oligosaccharides contain both lipophilic and hydrophilic residues. They are insoluble in chloroform/methanol/water at 3:2:1 but are solubilized in chloroform/methanol/water at 10:10:3 (20). When arsenite-challenged A375 cells were extracted with chloroform/methanol/water (3:2:1) and analyzed on one-dimensional SDS-PAGE, M14 was not solubilized (Fig. 7, lane 3). Upon further chloroform/methanol/water (10:10:3) extraction, however, a single radiolabeled band was detected that co-migrated precisely with M14 (Fig. 7, lane 4). Material that was insoluble in chloroform/methanol/water was free of M14 (Fig. 7, lane 5), as was the original Tris-solubilized material (data not shown).

Acid Hydrolysis of M14—Extracts of arsenite-challenged melanoma cells were treated with acid and analyzed on one-dimensional SDS-PAGE. Mild acid treatment caused the total loss of M14-associated radioactivity, whereas other mannosylated bands were unaffected (Fig. 8).
**Stress-induced Alterations in Glycosylation**

In a previous investigation (13), we described the induction of a 32-kDa protein (p32) in human normal and neoplastic cells stressed with sodium arsenite. The original objective of this study was to examine whether p32 was glycosylated and whether the extent of glycosylation was influenced by stress.

**M14** showed one major component slightly higher in molecular weight than the standard oligosaccharide Man$_5$GlcitolNAc (Fig. 9). The elution profile was similar to that observed for Glc$_3$Man$_5$GlcNAc$_3$ by others using identical conditions (21). Extracts from both control and stressed cultures displayed similar patterns of elution (Fig. 9).

**DISCUSSION**

In a previous investigation (13), we described the induction of an 32-kDa protein (p32) in human normal and neoplastic cells stressed with sodium arsenite. The original objective of this study was to examine whether p32 was glycosylated and whether the extent of glycosylation was influenced by stress. Preliminary experiments on cultures challenged with sodium arsenite (and other insults) and radiolabeled with [3H]mannose revealed that p32 was not detectably mannosylated in either control or stressed cells. However, under identical conditions, we observed in arsenite-treated cultures significantly increased incorporation of [3H]mannose into a component with an apparent molecular mass on one-dimensional SDS-PAGE of 14 kDa. We refer to this molecule as M14; and in this report, we provide an initial description of its induction, turnover, and stability in stressed cells and tentatively identify it as a lipid-linked oligosaccharide whose steady-state level is substantially enhanced in cultures exposed to noxious insult.

Expression of M14 in arsenite-challenged cells has not been reported previously. Several lines of evidence indicate that its induction is a stress-related event. Although sodium arsenite was the most potent inducer of M14, a broad range of other chemical insults, including a thiol-reactive reagent (iodoacetamide), heavy metals (zinc, copper, cadmium, and nickel), amino acid analogs (L-azetidine-2-carboxylic acid and L-canavanine), the calcium ionophore A23187, and disulfiram, increased significantly [3H]mannose incorporation into M14. These reagents also induced synthesis of the major stress proteins (13). Exposure of cells to auranofin (2,3,4,6-tetra-O-acetyl-L-thio-B-D-glucopyranosato-S-triethylphosphine gold(I) (Ridaura™)), an antiarthritic compound recently reported to induce the synthesis of stress proteins (22), also enhanced M14 expression in a manner similar to other tested agents.

Hyperthermia, the insult originally used to identify heat-shock (stress) proteins (1), also elevated M14 levels in A375 cultures allowed to recover from thermal insult. In addition, the kinetics of M14 induction and recovery paralleled those described previously for the major stress proteins (13). Peak expression of M14 occurred within 6-8 h of arsenite treatment, and recovery to pre-stress levels required approximately 8 h in arsenite-free medium.

Whereas these initial data suggested that M14 was a novel, glycosylated stress protein, studies with metabolic inhibitors demonstrated that this was not the case. Actinomycin D and cycloheximide failed to block M14 induction in arsenite-challenged cells, indicating that expression of this molecule was independent of both RNA and protein synthesis. Under identical conditions, induction of stress proteins was completely inhibited (13). In contrast, tunicamycin, which prevents N-linked protein glycosylation by inhibiting formation of lipid-linked oligosaccharides (23), completely blocked M14 induction. This observation, coupled with our inability to label metabolically M14 in control or stressed cultures with...
radioactive amino acids, suggested that M14 was not a stress-induced glycoprotein but a lipid-linked intermediate of protein glycosylation. The results of subsequent biochemical analyses on M14 were consistent with this hypothesis.

First, electrophoretically purified M14 was resistant to digestion with Pronase and peptide:N-glycosidase F. These data support the view that M14 is a glycosylated lipid and not a glycoprotein since peptide:N-glycosidase F hydrolyzes N-glycosidic bonds of glycoproteins (24). Resistance to Pronase provides additional support, although it should be noted that certain glycoproteins, particularly those which are heavily glycosylated or associated with fatty acids, are insensitive to Pronase treatment (25, 26).

Second, M14 sensitivity to digestion with endoglycosidase H indicates that the carbohydrate component of M14 is "high mannos" and suggests that it contains the minimal structure Man$_5$GlcNAc$_2$ since smaller lipid-linked species (Man$_n$GlcNAc$_2$) are endoglycosidase H-resistant (27, 28).

Third, the chase half-life of M14 from either control or stressed cultures was only 15 min, which compares to the reported half-life of other oligosaccharide lipid-linked intermediates (8–15 min) (29, 30).

Fourth, M14 was readily extracted from control and stressed cells with chloroform/methanol/water (10:10:3), a solvent frequently used to solubilize large, lipid-linked oligosaccharides (e.g., Glc$_3$Man$_3$GlcNAc$_2$-dolichol and Man$_4$GlcNAc$_2$-dolichol (31)). In contrast, M14 could not be extracted in a 3:2:1 mixture of the same solvent which readily solubilizes mono- and pyrophosphate derivatives of dolichol containing fewer saccharide moieties (31).

Fifth, M14 was acid-labile under conditions known to hydrolyze glycosyl-phosphate bonds in lipid-linked oligosaccharides (32), but to spare mannose" and suggests that it contains the minimal structure Glc$_3$Man$_3$GlcNAc$_2$-dolichol (31). In contrast, M14 could not be extracted in a 3:2:1 mixture of the same solvent which readily solubilizes mono- and pyrophosphate derivatives of dolichol containing fewer saccharide moieties (31).

Finally, other investigators (33–35), studying hepatocytes and oviduct membranes, have observed alterations in dolichol-linked oligosaccharides induced by steroids. Since steroids have also been shown to induce stress protein synthesis and thermal tolerance (6, 36, 37), these observations are consistent with our own and identify the lipid-linked oligosaccharides as potentially important mediators of the cellular response to stress and define a new component of cellular response to injury. One possible explanation for enhanced M14 levels in stressed cells is its decreased utilization due to inhibition of protein synthesis and thus lack of available "acceptor substrates." We consider this highly unlikely because under conditions in which sodium arsenite induced significantly increased levels of M14, protein synthesis was essentially unaffected. Similarly, cycloheximide blocked protein synthesis by more than 90% but failed to enhance M14 expression. Both pieces of evidence support the view that stress agents like sodium arsenite and amino acid analogs increase M14 levels by promoting its synthesis rather than blocking utilization.

Together, these data provide strong, although indirect, evidence that stressing of A375 melanoma cells can induce enhanced synthesis of large (six or more mannose moieties) lipid-linked oligosaccharides in addition to other well-documented changes in protein synthesis. Confirmation of these conclusions will require isolation of M14 in sufficient quantities for unequivocal structural determination.

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