Unique Asn-linked Oligosaccharides of the Human Pathogen 
Entamoeba histolytica

Received for publication, January 28, 2008, and in revised form, March 21, 2008. Published, JBC Papers in Press, April 16, 2008, DOI 10.1074/jbc.M800725200

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N-Glycans of Entamoeba histolytica, the protist that causes amebic dysentery and liver abscess, are of great interest for multiple reasons. E. histolytica makes an unusual truncated N-glycan precursor (Man₃GlcNAc₂), has few nucleotide sugar transporters, and has a surface that is capped by the lectin concanavalin A. Here, biochemical and mass spectrometric methods were used to examine N-glycan biosynthesis and the final N-glycans of E. histolytica with the following conclusions. Unprocessed Man₃GlcNAc₂, which is the most abundant E. histolytica N-glycan, is aggregated into caps on the surface of E. histolytica by the N-glycan-specific, anti-retroviral lectin cyanovirin-N. Glc₂Man₃GlcNAc₂, which is made by a UDP-Glc: glycoprotein glucosyltransferase that is part of a conserved N-glycan-dependent endoplasmic reticulum quality control system for protein folding, is also present in mature N-glycans. A swainsonine-sensitive α-mannosidase trims some N-glycans to biantennary Man₃GlcNAc₂. Complex N-glycans of E. histolytica are made by the addition of α1,2-linked Gal to both arms of small oligomannose glycans, and Gal residues are capped by one or more Glc. In summary, E. histolytica N-glycans include unprocessed Man₃GlcNAc₂, which is a target for cyanovirin-N, as well as unique, complex N-glycans containing Gal and Glc.

Entamoeba histolytica is the protist (single cell eukaryote) that causes millions of cases of amebic dysentery and liver abscess in regions where its fecal-oral spread cannot be prevented (1, 2). Asn-linked glycans (N-glycans) of E. histolytica are of great interest for seven reasons.

First, E. histolytica is missing many of the glycosyltransferases that make lipid-linked precursors to N-glycans and so makes a Man₅GlcNAc₂:PP-dolichol rather than Glc₂Man₅GlcNAc₂:PP-dolichol, which is present in most animals, plants, and fungi (3, 4). Second, E. histolytica N-glycans contribute to the quality control of protein folding in the endoplasmic reticulum (ER)² (4–6). In particular, E. histolytica has UDP-Glc:glycoprotein glucosyltransferase, calreticulin, glucosidase II, and ERGIC-53, which are the essential components of an N-glycan-dependent quality control system (4–6).

Third, the E. histolytica oligosaccharyltransferase (OST), which transfers N-glycans from the dolichyl pyrophosphate-linked precursor to Asn on the nascent peptide in the lumen of the ER is composed of four subunits rather than seven or eight subunits found in metazoan, fungi, and plants (7). As a result, the E. histolytica OST has different kinetics than the OSTs of higher eukaryotes (8). As well, the E. histolytica OST prefers to transfer N-glycans, which resemble its own (Man₅GlcNAc₂), rather than the longer N-glycans of metazoa and fungi (Glc₂Man₅GlcNAc₂) (8).

Fourth, E. histolytica has a limited set of nucleotide sugar transporters (UDP-Gal and UDP-Glc), which transport activated sugars into the lumen of the ER and Golgi (9). UDP-Gal and UDP-Glc are used to make unique O-phosphodiester-linked glycans of E. histolytica proteophosphoglycans (10) and may be used to make complex N-glycans.

Fifth, E. histolytica causes disease when the protist uses a Gal- and GalNAc-binding lectin on its surface to adhere to mucins on the surface of host colonic epithelial cells and then lyses host cells by means of secreted proteases and pore-forming peptides (1, 2, 11, 12). Heavy subunits of the E. histolytica Gal/GalNAc lectins have 7–14 potential sites for N-linked glycosylation, and inhibition of N-glycan synthesis results in a Gal/GalNAc lectin that is unable to bind its target (11).

Sixth, the plant lectin concanavalin A binds to glycoproteins on the surface of E. histolytica and aggregates them into caps that are shed into the medium (13). This result suggests the possibility that E. histolytica may have on its surface high mannose N-glycans. In a similar way, gp120 of HIV has on its surface N-glycans composed of Man₅GlcNAc₂ (14). These high mannose N-glycans on gp120 are the target of an anti-HIV human monoclonal antibody (2G12) and of bacterial anti-retroviral lectins (cyanovirin-N and scytovirin) (15, 16).

¹This work was supported, in whole or in part, by National Institutes of Health Grants AI 44070 (to J. S.), GM 43768 (to R. G.), P41 RR10888 and S10 RR15942 (to C. E. C.), and GM 31318 (to P. W. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

²The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1, Figs. 1–5, and additional references.

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The abbreviations used are: ER, endoplasmic reticulum; MS, mass spectrometry; HPAEC, high performance anion exchange chromatography; PBS, phosphate-buffered saline; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; MES, 4-morpholineethanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Hex₃, Hex₄GlcNAc₂; NYT, N₂-acetyl-N₁25Tyr-Thr-NH₂; OST, oligosaccharyltransferase; PNGase F, peptide/N-glycanase F; HPLC, high pressure liquid chromatography.
Seventh, although N-linked glycans of *Giardia lamblia* and kinetoplastids (e.g. *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania mexicana*) have been characterized, N-glycans of the vast majority of protists (e.g. *E. histolytica*, *Trichomonas vaginalis*, *Plasmodium falciparum*, *Toxoplasma gondii*, and *Cryptosporidium parvum*) remain uncharacterized (3, 17–19). Characterization of *E. histolytica* N-glycans then may lead to a better understanding of the diversity of N-linked glycosylation in protists and may identify novel sugar linkages that have not previously been identified in higher eukaryotes.

Here, biochemical methods including in vivo and in vitro labeling, as well as mass spectrometry, were used to characterize *E. histolytica* N-glycans. *E. histolytica* N-glycans include unprocessed Man₅GlcNAc₂, which is a target for cyanovirin-N, as well as unique, complex N-glycans containing Gal and Glc.

**EXPERIMENTAL PROCEDURES**

**Bioinformatic Predictions**—The predicted proteins of the *E. histolytica* genome, which has been extensively sequenced, were searched with BLASTP and representatives (e.g. *Saccharomyces*, *Homo*, or *Escherichia*) of each of 79 glycosyltransferase families and 104 glycosylhydrolase families present in the data base of carbohydrate-active enzymes (CAZy) (20–22). Putative *E. histolytica* glycosyltransferases and glycosylhydrolases were compared with proteins in the NR Database and to the conserved domain data base at the NCBI using PSI-BLAST. Signal peptides and transmembrane helices of *E. histolytica* proteins were predicted using SignalP and TMHMM, respectively (23, 24).

**Reagents**—[2-³H]Man (30 Ci/mmol), [U-¹⁴C]Glc (200 mCi/mmoll), UDP-[³H]Gal (5.8 Ci/mmoll), and UDP-[³H]Glc (300 mCi/mmoll) were from American Radiolabeled Chemicals (St. Louis, MO). Peptide:N-glycanase F (PNGase F) was from New England Biolabs. Jack bean α-mannosidase and almond β-glucosidase were from Sigma. Coffee bean α-galactosidase, β1,3,4,6-galactosidase, and β-galactosidase II (β-galactosidase and β-glucosidase activities) were from Calbiochem. *Aspergillus saitoi* α1,2-mannosidase, bovine testes β-galactosidase, jack bean hexosaminidase, Man₃GlcNAc₂, and Man₃GlcNAc₂ standards were from Glyko (San Leandro, CA). Maltase and amyloglucosidase (α-glucosidases) were from Roche Applied Science. Golgi endomannosidase (25) was a generous gift from Dr. Robert Spiro (Harvard Medical School). Cyanovirin-N and
scytovirin were generous gifts of Barry O'Keefe (NCI-Frederick, National Institutes of Health).

In Vivo Labeling Conditions—E. histolytica strain HM1:IMSS was grown axenically in TYI-S-33 medium containing 0.1% (w/v) Glc to minimize the storage of glycogen, in which fragments otherwise contaminate N-glycan extracts (26). E. histolytica cultures from five 32 cm² flasks were combined, centrifuged, and transferred to a microcentrifuge tube with 0.5 ml of radiolabeling medium (the same as described above, excluding Glc and containing 200 μCi of [2-3H]Man or 2 mCi of [14C]Glc) (3). Amebas were incubated for the indicated time at 37 °C and then washed in PBS several times before processing.

Extraction of N-Glycans—Non-incorporated radiolabel was removed by washing the cell pellet with 50% methanol. Cells were lysed with a Dounce homogenizer in 4 ml of Tris-HCl buffer, pH 8. To reprecipitate proteins, the pH was adjusted to 5 with acetic acid, and 4 ml of methanol was added. Tubes were chilled at −20 °C for 4 h and centrifuged at 14,000 rpm for 10 min. This step was repeated twice to wash out soluble glycans such as glycogen fragments. The methanol-denatured protein pellet was dispersed and treated with 5,000 units of PNGase F in acetate buffer, pH 5, at 37 °C for 16 h. The suspension containing the released N-glycans in solution was adjusted to 50% methanol to precipitate large carbohydrates and proteins. After 4 h at −20 °C, the supernatant was cleared by centrifugation and dried. Oligosaccharides were further purified on a porous graphitic carbon Hypercarb column (Thermo Keystone) and washed with 3 ml of distilled water. The N-glycans, which were eluted with 30% acetonitrile, 0.1% trifluoroacetic acid, were dried and resuspended in 300 μl of water.

In Vitro Labeling of N-Glycans—Intact E. histolytica vesicles from freshly harvested cultures were prepared as described for nucleotide sugar transport assays (9), excluding separation ER enriched vesicles from light Golgi enriched vesicles. Labeling of endogenous acceptors was performed by incubating the E. histolytica vesicles with UDP-[3H]Gal and UDP-[14C]Glc as

FIGURE 2. The complexity of N-glycans made by E. histolytica trophozoites increases dramatically with time. E. histolytica trophozoites were incubated with [2-3H]Man for 10, 20, 60, and 150 min, and N-glycans were released with PNGase F and separated by Bio-Gel P-4 filtration (A–D), where Hex, indicates their size. Isomers with the same number of hexoses were further isolated on an HPAEC-PA100 column (E–H), with the retention time (ret.; min) shown in italic and the identifier name from Fig. 1 (e.g. H5.1) underlined below. After 10-min labeling (A and E), the predominant E. histolytica N-glycans were unmodified Man5GlcNAc2, and the glucosylated product of UDP-Glc:glycoprotein glucosyltransferase (Glc-Man5GlcNAc2) (H6.1). As shown in the inset in A, Glc5Man5GlcNAc2 is by far the most abundant N-glycan in the presence of the glucosidase II inhibitor castanospermine (CTS). After 20-min labeling (B and F), two mannosidase digestion products are apparent: Man5GlcNAc2 (H4.1) and Man5GlcNAc2 (H3.1). After 60-min labeling (C), complex N-glycans are apparent in Hex, and Hex, pools. After 150-min labeling (D and G), novel, complex N-glycans are present in all of the pools (see further characterization in Fig. 4). frct., fraction.
described (9). The vesicle preparation still carried a significant amount of cytosolic epimerase activity, which converts UDP-Gal to UDP-Glc, in a reversible reaction. Because of this activity, \( ^{3}H\)Glc was incorporated into *E. histolytica* glycoproteins when labeling with UDP-[\(^{3}H\)]Gal and vice-versa. Therefore, specific labeling was not possible. The labeling of glycans was started by mixing 500 \( \mu l \) of vesicle suspension and 500 \( \mu l \) of reaction buffer (10 mM MnCl\(_2\), 10 mM MgCl\(_2\), 10 mM CaCl\(_2\), 0.5 M sucrose, 30 mM triethanolamine, pH 7.2, 2 \( \mu M \) UDP-Gal, and 5 \( \mu M \) UDP-Glc and 30 \( \mu Ci \) of each radiolabeled precursor). After 45 min at 37 °C, the reaction was diluted with 1 volume of 100 \( \mu M \) UDP-Gal and UDP-Glc and separated into 100-\( \mu l \) aliquots, each of which was precipitated with 900 \( \mu l \) of 4% perchloric acid. After chilling on ice for 30 min, the preparation was centrifuged at 16,000 \( \times \) g for 30 min, and the pellets were combined and neutralized. The pellets were resuspended with 2 ml of 10 mM Tris-HCl buffer, pH 8, in 50% methanol and homogenized in a 3-ml Dounce. After 2 h at \(-4^\circ C\), the suspension was centrifuged at 14,000 \( \times \) g for 10 min, and the pellet was washed twice with Tris-HCl buffer, pH 8, to remove traces of methanol prior to release of N-glycans by digestion with PNGase F (see conditions above).

**Sample Preparation for Mass Spectrometry**—The starting material to isolate N-glycans for mass spectrometry was a cell pellet obtained from 60 flasks of *E. histolytica* culture. First, lipids were extracted three times with 30 ml of chloroform: methanol:water (10:10:3). Soluble carbohydrates (mostly glycan fragments) were removed by extracting three times with 20 ml of 10 mM Tris-HCl buffer, pH 8, and reprecipitating with 1 volume of chilled methanol for 4 h at \(-20^\circ C\). After a final wash in buffer, the pellet was digested with 1 \( \mu g \) of TPCK-treated trypsin in 1 ml of 10 mM Tris-HCl buffer, pH 8, for 16 h at 37 °C. After boiling to inactivate the protease, the suspension was digested with 20 \( \times \) 10\(^3\) units of PNGase F in the presence of phenylmethylsulfonyl fluoride for 16 h at 37 °C. The mixture was precipitated with 50% methanol, and the supernatant was purified through a porous graphite column, followed by passage through a 3-ml Amberlite mixed bed ion exchange column (H\(^+\)/acetate form). Finally, the N-glycan extract was dried and resuspended in 300 \( \mu l \) of water.

**Chromatography**—A Bio-Gel P-4 superfine mesh column of 1 \( \times \) 120 cm was equilibrated in 0.1 M acetic acid, 1% n-butyl alcohol. 300 \( \mu l \) of glycan sample was applied and run at a constant flow rate of 5 ml/h, and 1.3-ml fractions were collected. Radioactivity was determined by liquid scintillation counting. The distribution coefficient \( K_d = (V_e - V_o)/(V_t - V_o) \) was determined using the glycan elution volume \( V_e \) relative to the elution of cytochrome C (marker for the exclusion volume; \( V_o \)) and glucose (marker for the total volume; \( V_t \)). GlcNAC, diacytelchitobiase, and radiolabeled Man\(_5\)GlcNAC\(_2\) (prepared from the dolichol-linked precursors of \( \Delta 9 \)Saccharomyces cerevisiae (4)) were used to calibrate the system. For non-radiolabeled samples, peaks were collected according to the \( K_d \) of equivalent radiolabeled specimens.

To separate isomers with the same molecular mass, fractions obtained by gel filtration were separated by high performance anion exchange chromatography (HPAEC) with a pulse amperometric detector in a Dionex LC20 instrument through a PA100 column (250 \( \times \) 4 mm) equilibrated in 150 mM NaOH. The flow rate was 0.6 ml/min with a sodium acetate gradient from 12.5 mM (0–3 min) to 50 mM (at 31 min) and finally to 175 mM (at 70 min). A desalter membrane was installed to remove sodium ions, neutralizing the carbohydrate-containing eluent. Fractions of 0.3 ml were collected, and aliquots were taken for scintillation counting. The retention time of each isomer was determined. The system was calibrated with known N-glycans (Man\(_5\)GlcNAC\(_2\), Man\(_4\)GlcNAC\(_2\), and Man\(_3\)GlcNAC\(_2\) prepared from \( \Delta 9 \)S. cerevisiae cells) and with the series laminaribiose, laminaritriose, and laminaratetraose (routinely used as internal markers). Non-radiolabeled samples for mass spectrometry were isolated on a 250 \( \times \) 9 mm semi-preparative PA100 column (flow rate of 1.5 ml/min, collecting 0.7-ml fractions) calibrated and operated (excluding the internal markers) as described above. Carbohydrate-containing peaks were detected by a pulse amperometric detector, and the corresponding fractions were pooled and dried.

**Ion Exchange Mini-columns**—The resins used were Dowex 50 (H+ form), Dowex 1 (formate form), and Amberlite mixed bed (IRA-400 H+ form and IR-120 acetate form). Columns (0.5 \( \times \) 2.5 cm) were equilibrated in deionized water. The glycan sample (0.5 ml) was applied followed by water washes, and fractions of 0.25 ml were collected for scintillation counting. The retention behavior of glycans was compared with neutral or charged standards (2-\( ^{2}H \))Man, \( [^{3}H] \)GlcN, \( [^{3}H] \)Glc 1-phosphate, and UDP-[\(^{3}H\)]Glc.

**FIGURE 3.** The abundance of Glc in complex N-glycans of *E. histolytica* trophozoites increases with their length. *A*, *E. histolytica* N-glycans labeled with [U-\(^{14}C\)]Glc and separated by Bio-Gel P-4 filtration. The number of hexoses in each glycerm is indicated (Hex\(_n\)), according to calibrated standards prepared from Man\(_5\)GlcNAc\(_2\), digested with jack bean \( \alpha \)-mannosidase and A. saitoi \( \alpha 1,2 \)-mannosidase. *B*, relative abundance (weight percentage) of neutral sugars for each peak after acetic hydrolysis, as determined by HPAEC chromatography. frac., fraction.
Glycosidase Digestions—The primary enzime used for the glycosidase digests was jack bean α-mannosidase (37), which removes the Man\(_{9-11}\)GlcNAc\(_2\) residual fragment 11.5, which was fully characterized in a separate [U-14C]Glc-labeling experiment. Note that jack bean α-mannosidase (UBAM) removes an exposed Man\(_9\) (as in isomer H4.3), but it does not remove the Man\(_{10}\) unless the Man\(_{11}\) arm is digested first. Hence, isomer H4.2 becomes susceptible to jack bean α-mannosidase digestion only after removal of Gal\(_{1,2}\)-by α-galactosidase. The mode of action of jack bean α-mannosidase (37) supports our assumption that the different sensitivities of H4.2 and H4.3 are due to accessibility of α-linked Man residues. In accordance with this, the underlying structures of other isomers were inferred as well. ASAM, A. saitoi α,1,2-mannosidase.

**Glycosidase Digestions**—All the glycosidase digests were performed in a volume of 100 μl for 16 h at 37 °C. The conditions were adjusted by digestion of known glycan to assure a complete reaction. A final concentration of 100 mM potassium phosphate buffer, pH 6.5, was used for coffee cans to assure a complete reaction. A final concentration of 100 mM sodium acetate buffer, pH 4.8, was used for Jack bean α-galactosidase, maltase, and Amyloglucosidase; 100 mM sodium acetate buffer, pH 4.8, was used for Jack bean α-acetyl Man/GlcNAc2. For isomers H6.2, H6.5, and H7.1, the r.t. and digestion products were identified according to retention times (r.t.) of known standards (e.g., Man\(_9\)GlcNAc\(_2\)).

H.3.1 r.t. 6 min Man\(_9\)GlcNAc\(_2\)
H.3.1 r.t. 6.5 min Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.4.1 r.t. 9 min Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.4.2 r.t. 11.5 min Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.4.3 r.t. 10.5 min Gal\(_1\)Man\(_9\)GlcNAc\(_2\)

H.5.1 r.t. 12 min Man\(_9\)GlcNAc\(_2\)
H.5.2 r.t. 17 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.5.3 (r) r.t. 19 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.5.4 r.t. 14.5 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)

H.6.1 r.t. 22 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.6.2 r.t. 22 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.6.3 r.t. 23 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.6.4 r.t. 24 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.6.5 r.t. 24 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)

H.7.1 r.t. 26 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)
H.7.2 r.t. 28 min Glc\(_4\)Gal\(_1\)Man\(_9\)GlcNAc\(_2\)

**FIGURE 4. Characterization of the E. histolytica N-glycans by in vivo labeling and glycosidylhydrolyse digests.** E. histolytica N-glycans, which were prepared as in Fig. 2, were treated with glycosylhydrolases, and digestion products were identified according to retention times (r.t.) of known standards (e.g., Man\(_9\)GlcNAc\(_2\)).

**Monosaccharide Composition**—Radiolabeled samples were dried, resuspended in 300 μl of 2 N HCl in microcentrifuge tubes flushed with N\(_2\) to minimize oxidation, and hydrolyzed for 2 h at 90 °C. Acid was removed by several rounds of evaporation under N\(_2\). The neutral sugars were separated by HP ACEC on a MA1 250 × 4 mm column (flow rate of 0.4 ml/min, collecting 0.2-ml fractions) with an NaOH gradient from 100 mM (0–3 min) to 850 mM (to 45 min). GlcN, Glc, Man, and Gal were used as internal standards.

**Mass Spectrometry**—Oligosaccharides were permethylated as described previously (27). MALDI-TOF MS was performed on a Bruker Reflex IV mass spectrometer in positive reflectron mode. Between 20 and 50 pmol of sample dissolved in 20% acetonitrile was applied to the MALDI target with an equal volume of 2,5-dihydroxybenzoic acid (20 mg/ml) in 20% acetonitrile with 10 mM sodium acetate added as a cation source. The spectra resulting from 150 and 200 shots from a 337-nm nitrogen laser were summed. The laser pulse was 3 ns. Collision-induced dissociation fragmentation data were collected using a QStar Pulsar i quadrupole orthogonal time-of-flight mass spectrometer (Applied Biosystems Inc., Framingham, MA) equipped with an electrospray ionization source. Capillaries were pulled to a 1-micron orifice diameter. Argon (3 p.s.i.) was used as the collision gas for MS/MS experiments. The range of operator-controlled collision voltages was 35–90 V. Between 2 and 3 pmol of sample were consumed during each MS/MS experiment. The mass spectrometer parameters were as follows for MS/MS experiments: DP1, 75.0 V; FP, 245.0 V; DP2, 30.0 V; CG, 3.0 p.s.i.; IRD, 6.0 V; IRW, 5.0 V; GS1, 5.0 p.s.i.; GS2, 10.0 p.s.i.; and CUR, 12.0 p.s.i. The ion spray voltage was between 4,000 and 4,500 V. For pseudo MS/MS experiments, the following parameters were used: DP1, 130.0 V; FP, 300.0 V; DP2, 40.0 V; CG, 3.0 p.s.i.; IRD, 6.0 V; IRW, 5.0 V; GS1, 5.0 p.s.i.; GS2, 10.0 p.s.i.; and CUR, 12.0 V. Nomenclature is that of Domon and Costello (28) unless otherwise indicated.

**In Vitro OST Assay**—Total cellular membranes of E. histolytica were incubated for 2–90 min at 37 °C with the membrane-permeable tripeptide acceptor 5 μM N\(^{ε}\)-acetyl-N\(^{\text{2,23}}\)-Tyr-Thr-NH\(_2\) (NYT) in the presence of deoxynojirimycin to ensure that the glycopeptide products were not degraded by glucosidases I and II (29, 30). In some experiments, swainsonine was added to determine the effect of endogenous E. histolytica mannosidases on glycopeptides. Glycopeptide products were collected by binding to immobilized concanavalin A and separated by HPLC using an aminopropyl silica column. Glycopeptide standards (Man\(_9\)GlcNAc\(_2\)-NYT and Man\(_9\)GlcNAc\(_2\)-NYT) were prepared using the Saccharomyces OST and purified dicholochol-linked oligosaccharides (31).
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tolytica were immediately fixed with 2% paraformaldehyde in PBS for 10 min at 4 °C. To determine whether cyanovirin-N aggregates and forms caps on the surface of E. histolytica, we incubated cyanovirin-N-bound cells in PBS for 15 min at 37 °C and subsequently fixed them in 2% paraformaldehyde. For labeling the internal membranes and surface of E. histolytica, amebas were fixed in 2% paraformaldehyde and 0.1% Triton X-100. Organisms were visualized with a DeltaVision deconvoluting microscope (Applied Precision, Issaquah, WA) with the help of Landon Moore of the Department of Genetics and Genomics at Boston University School of Medicine. Images were taken at ×100 primary magnification and deconvolved using Applied Precision softWoRx software.

RESULTS

Brief Summary of the Experimental Strategy—The structures of E. histolytica N-glycans, which are shown in Fig. 1, were determined by in vivo labeling with [2-3H]Man (shown in Fig. 2) or [U-14C]Glc (shown in Fig. 3) or by the separation of N-glycans released by PNGase F on Bio-Gel P-4 and HPAEC, followed by digestion with specific glycosylhydrolases (shown in Fig. 4). Alternatively, E. histolytica N-glycans were identified by in vitro labeling of E. histolytica membranes with nucleotide sugars (nucleotide sugar transfer assays shown in Fig. 6) or with a radiolabeled tripeptide (OST assays shown in Fig. 7). In addition, E. histolytica N-glycans were examined by mass spectrometry (shown in Figs. 8 and 9). These strategies allowed us to identify each E. histolytica N-glycan by multiple methods. Finally, we localized unprocessed N-glycans on the surface of E. histolytica using the anti-retroviral lectin cyanovirin-N (shown in Fig. 5).

The Most Abundant N-Glycan of E. histolytica Is Unprocessed Biosynthetic ManN,N,GlcNAc2—The most abundant N-glycan of E. histolytica after in vivo labeling for 20–150 min with [3H]Man or for 3 h with [14C]Glc (Figs. 2 and 3) was unprocessed, biosynthetic ManN,GlcNAc2 (H5.1 in Fig. 1), which was characterized by mannosidase digestions (Fig. 4). When E. histolytica were pulse-labeled for 10 min with [2-3H]Man, washed, and chased for 3 h with unlabeled Man, biosynthetic ManN,GlcNAc2 was still the most abundant N-glycan (data not shown). This result shows that many E. histolytica N-glycoproteins are not modified by Golgi-type glycosylhydrolases and glycosyltransferases.

ManN,GlcNAc2 on the Surface of E. histolytica Trophozoites Is Bound and Capped by the Anti-retroviral Lectin Cyanovirin-N—The presence of biosynthetic ManN,GlcNAc2 (H5.1) on the surface of cultured E. histolytica was demonstrated using the anti-retroviral lectin cyanovirin-N (Fig. 5) (15). Cyanovirin-N is specific for the ManN,GlcNAc2 (E. histolytica N-glycans) or on three arms of ManN,GlcNAc2 (N-glycans of gp120) (Fig. 5A). Cyanovirin-N was bound to the surface and vesicular membranes of fixed and permeabilized E. histolytica (Fig. 5B). Cyanovirin-N was also bound evenly to glycoproteins on the surface of chilled but living E. histolytica (Fig. 5C). When E. histolytica were warmed to 37 °C, cyanovirin-N aggregated into caps (Fig. 5D), which resemble those formed by the lectin concanavalin A on the E. histolytica surface (11, 13). As a negative control, scytovirin, another anti-retroviral lectin that is specific for Man on the upper (D3) arm of ManN,GlcNAc2, which is absent from E. histolytica N-glycans, did not bind to the surface or vesicles of E. histolytica (data not shown) (15).

GlcnManN,N,GlcNAc2, the Product of UDP-Glc:Glycoprotein Glucosyltransferase Involved in N-Glycan-dependent Quality Control of Protein Folding Is Also Present in Mature N-Glycans of E. histolytica—At the earliest time points of metabolic labeling with [2-3H]Man (Fig. 2A), E. histolytica N-glycans were composed of ManN,GlcNAc2 (H5.1) and Glc1ManN,GlcNAc2 (H6.1). Glc1ManN,GlcNAc2 was markedly increased, whereas ManN,GlcNAc2 was decreased, when E. histolytica trophozoites were labeled in the presence of castanospermine, which is a glucosidase II inhibitor (inset in Fig. 2A) (30). These results are consistent with the presence of all the components of N-glycan-dependent quality control of protein folding in E. histolytica (4–6). See supplemental Table 1 for a list of the E. histolytica glycosylation-related genes.

When E. histolytica membranes were incubated in vitro with UDP-[3H]Glc, the most abundant N-glycan was also Glc1ManN,GlcNAc2 (Fig. 6, B and E). Glc1ManN,GlcNAc2 was also present in relatively high amounts in mature glycoproteins, indicating that this N-glycan is not just a transient species in E. histolytica (Fig. 2, D and H).

Some E. histolytica N-Glycans Are Trimmed Back to Man5GlcNAc2 and Man4GlcNAc2 by a Swainsonine-sensitive Mannosidase—The activity of the E. histolytica α-mannosidase was inferred by the accumulation of Man5GlcNAc2

FIGURE 5. The anti-retroviral lectin cyanovirin-N, which recognizes high Man N-glycans (15, 16), binds to the surface E. histolytica trophozoites and forms caps. A, cyanovirin-N binds to terminal α1,2-linked Man, which is present on each of the three arms of ManN,GlcNAc2, and is also present on the single arm of ManN,GlcNAc2. B, cyanovirin-N binds to the surface and to vesicular membranes of fixed and permeabilized E. histolytica. n, nucleus. C, cyanovirin-N is evenly distributed on the surface of a live E. histolytica incubated with the lectin in the cold, so it cannot cap. D, cyanovirin-N is capped on the surface of a live E. histolytica that is allowed to warm up to 37 °C for 10 min. The negative control was scytovirin (a lectin that binds to an intact D3 arm, which is absent in E. histolytica N-glycans). E. histolytica incubated with scytovirin were not labeled (data not shown).
after labeling E. histolytica in vivo for longer times (20–150 min) with [3H]Man (Fig. 2). Man₄GlcNAc₂-NYT and Man₃GlcNAc₂-NYT were also present after 2–30 min when intact E. histolytica membranes were incubated with the OST substrate NYT (Fig. 7) (10, 29, 31). The E. histolytica mannosidase activity was inhibited by swainsonine, which has been used previously to inhibit class II mannosidases (Fig. 7) (30). In contrast, the E. histolytica mannosidase was not inhibited by deoxymannojirimycin, which inhibits class I mannosidases that are absent from E. histolytica (data not shown).

The largest oligosaccharide attached to the radiolabeled tripeptide (NYT) in vitro in the presence of UDP-Gal, UDP-Glc, and swainsonine is Glc₃Man₄GlcNAc₂ (H₆.1) (6). These results suggest that the OST, the mannosidase, and the UDP-Glc:glycoprotein glucosyltransferase are all present in the ER, whereas the Gal- and Glc-transferases (see below) are in a distinct organelle (likely the Golgi apparatus).

**Elongation of E. histolytica Complex N-Glycans**

- Initiated by Addition of α-Linked Gal to Either Arm of Man₃–5GlcNAc₂—E. histolytica membranes incubated with UDP-[³H]Gal or UDP-[³H]Glc, the two nucleotide sugars that are transported by E. histolytica membranes (9), produced abundant products ranging from Hex₄GlcNAc₂ (Hex₄) to Hex₇ (Fig. 6). In contrast, radiolabeled products were not formed when E. histolytica membranes were incubated with radiolabeled GDP-Man or...
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GDP-Fuc (data not shown). Indeed, monosaccharide analysis of N-glycans of *E. histolytica* labeled in vivo with [14C]Glc revealed Man, Gal, and Glc but did not show other hexoses or deoxyhexoses (Fig. 3B). Treatment with hexosaminidase, along with mass spectrometry results, showed that N-glycan extensions do not contain hexosamines. All of the N-glycans of *E. histolytica* were neutral oligosaccharides (data not shown) lacking charged sugars (e.g. sialic acids) or other modifications such as sulfation or phosphorylation.

As shown by sensitivity to α-galactosidase but not to β-galactosidase, a single α-linked Gal was added to the terminal Man on either or both arms of Man3–5GlcNAc2 when *E. histolytica* were labeled in vivo or in vitro (e.g. H3.2, H4.3, H5.2, H5.4, and H6.4) (Figs. 3 and 4). Fig. 8B shows the signature of a terminal 1,2-linked hexose (a 1,3A2 fragment that cannot originate from Man3GlcNAc2) (H3.1). This result indicates that the minor Hex3 isomer (H3.2) is indeed elongated with an α,1,2-linked Gal. The spectrum for Hex5 N-glycans (Fig. 9) also shows the cross-ring 1,3A2 fragment, indicating the same Gal configuration (e.g. H6.3 to H6.5).

In Many Complex N-Glycans of *E. histolytica*, Gal Is Capped by One or More Glc Residues—The evidence for complex *E. histolytica* N-glycans containing Gal and Glc included the following. With increasing time, Hex10 to Hex10 were present in N-glycans of *E. histolytica* labeled in vivo with [2-3H]Man (Fig. 2) or [14C]Glc (Fig. 3). As the size of N-glycans increased, the percentage of Glc in the oligosaccharides increased (Fig. 3B), and these larger N-glycans (e.g. H5.3, H6.2, H6.5, and H7.1) were resistant to α- and β-galactosidases, consistent with a Glc cap (Fig. 4). With the exception of the product of the UDP-Glc:glycoprotein glucosyltransferase (Glc5Man5GlcNAc2) (H6.1), Glc was only added to N-glycans of *E. histolytica* after the addition of Glc caps (Fig. 6).

Because the complex N-glycans of *E. histolytica* that have Glc caps were resistant to all the glycosylhydrolases that we tested, it was not possible to determine whether the Glc-extensions are α- or β-linked; hence, the configuration of the linkage between Glc and Gal remains to be defined. A pseudo MS/MS experiment performed on B3 ions of Hex5 (which originate from glycans with a linear series of four or more hexoses) suggests the possibility that some of the extending Glc residues are 1,6-linked (supplemental Fig. 4). A full discussion of the mass spectrometry experiments is presented in the supplemental material.

**DISCUSSION**

Properties of *E. histolytica* N-Glycans That Distinguish Them from N-Glycans of Fungi and Metazoa—As shown by analysis of predicted proteins from whole genome sequences, *E. histolytica* is missing seven asparagine-linked glycosylation enzymes, and so N-glycans are built upon the truncated precursor Man5GlcNAc2 (H5.1 in Fig. 1) (see supplemental Table 1) (3). Man5GlcNAc2 is glycosylated by the UDP-Glc:glycoprotein glucosyltransferase of the N-glycan-dependent quality control system for protein folding to make Glc5Man5GlcNAc2 (H6.1) (4–6). For the same reason, N-gly-
cans of *T. vaginalis*, the cause of vaginitis, are also built upon Man$_5$GlcNAc$_2$, which is again glucosylated to Glc$_1$Man$_5$GlcNAc$_2$ (3, 6).

In contrast to fungi and metazoa, a large proportion of *E. histolytica* N-glycans reach the plasma membrane as unmodified Man$_5$GlcNAc$_2$ (H$_5.1$), bypassing ER and Golgi mannosidases and glycosyltransferases. Similarly, a large fraction of mature N-glycans of *E. histolytica* are composed of Glc$_1$Man$_5$GlcNAc$_2$, as has been described in *Leishmania* (33). The presence of Glc$_1$Man$_5$GlcNAc$_2$ in mature glycoproteins suggests that it may have other functions in addition to serving as an intermediate in the N-glycan-dependent quality control of folding in the ER lumen (4–6).

Because we did not link N-glycans to sites on particular *E. histolytica* glycoproteins, there are three possible explanations for the abundance of unmodified Man$_5$GlcNAc$_2$ in the plasma membrane. First, N-glycans on some *E. histolytica* glycoproteins are never processed. Second, N-glycans at multiple sites on the same glycoprotein are processed differently. Third, *E. histolytica* mannosidases and glycosyltransferases that make
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complex N-glycans are very inefficient. We speculate that the high Man N-glycans of *E. histolytica* may be harder to recognize by the host immune system than the unique complex N-glycans of *E. histolytica*. A similar argument has been made to explain the abundance of high Man N-glycans on gp120 of HIV (14).

The capping of *E. histolytica* plasma membrane glycoproteins containing Man$_{3}$GlcNAc$_{2}$ by cyanovirin-N suggests the possibility that anti-viral lectins such as cyanovirin-N might be used to block binding of *E. histolytica* to the host epithelium. Cyanovirin-N inhibits phagocytosis of mucin-coated beads by *E. histolytica*.\(^3\) As well, affinity columns with concanavalin A efficiently capture *E. histolytica* glycoproteins with N-glycans (so-called N-glycome) for mass spectrometric identification of peptides.\(^3\)

In addition to OST and UDP-Glc-glycoprotein glucosyltransferase activities (6, 8), the ER of *E. histolytica* contains a swainsonine-inhibitable α-mannosidase, which trims a fraction of the N-glycans to Man$_{3}$GlcNAc$_{2}$ (H3.1) and Man$_{4}$GlcNAc$_{2}$ (H4.1). As in metazoa, the trimmed N-glycans of *E. histolytica* are building blocks for formation of complex N-glycans (34, 35).

*E. histolytica* has a single predicted α-mannosidase (glyco- sylhydrolase family 92 or GH92), which is similar to those of many Gram-positive bacteria (*e.g.* *Streptococcus, Porphyromonas, Bacteroides, and Mycobacteria*) (supplemental Table 1) (20–22). Phylogenetic analyses strongly suggest that the *E. histolytica* GH92 mannosidase was obtained by lateral gene transfer from bacteria, and this clade is supported with high bootstrap values (20–22, 32). Phylogenetic analyses strongly suggest that the *E. histolytica* GH92 mannosidase was obtained by lateral gene transfer from bacteria, and this clade is supported with high bootstrap values (20–22, 32).

The N-glycans of *E. histolytica*, which have α1,2-linked Gal that is extended with Glc, are simpler than the complex N-glycans of metazoa. The N-glycans of *E. histolytica* are consistent with the experimental demonstration of two *E. histolytica* nucleotide sugar transporters (for UDP-Glc and UDP-Gal) (9) and with bioinformatic predictions of a limited number of Golgi glycosyltransferases in *E. histolytica* (see supplemental Table 1). O-phosphodiester-linked glycans of *E. histolytica* are also composed of Gal capped with Glc, but the linkages are different from those of N-glycans (10). Although α1,2-Gal linked to Man is also present in N-glycans of *Schizosaccharomyces pombe* (36), N-glycans with distal Glc capping residues are not present in the human host of *E. histolytica*. This suggests the possibility that the unique complex N-glycans of *E. histolytica* may be targets for future anti-amebic vaccines. In support of this idea, the O-phosphodiester-linked glycans, which are also rich in Gal and Glc, are one of a limited number of current anti-amebic vaccine targets (1, 2, 10).

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3. D. Ratner and J. Samuelson, unpublished observations.