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IDENTIFICATION OF *N*-ACETYLGLUCOSAMINE-α-1-PHOSPHATE TRANSFERASE ACTIVITY IN *DICTYOSTELIUM DISCOIDEUM*: AN ENZYME THAT INITIATES PHOSPHOGLYCOSYLATION

Hudson H. Freeze* and Mie Ichikawa

La Jolla Cancer Research Foundation
10901 North Torrey Pines Road
La Jolla, California 92037

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SUMMARY- A lysosomal proteinase from *Dictyostelium discoideum* was previously shown to have GlcNAcα-1-P residues in phosphodiester linkage to serine. We have identified a GlcNAc-α-1-P transferase activity in membrane preparations using UDP[^{3}H]GlcNAc and a peptide acceptor with three tandem Ser-Gly repeats. We established an assay, proved the structure of the product, determined the Kms for donor and acceptor and showed that the glycopeptide binds a GlcNAc-α-1-P specific rabbit antibody. These findings provide the tools to search for mutants lacking GlcNAc-α-1-P transferase activity as a probe for the function of this modification we call phosphoglycosylation.

Dictyostelium discoideum makes phosphorylated *N*-linked oligosaccharides. The first step in this pathway is the addition of GlcNAc-α-1-P to the 6-position of selected mannose residues (reviewed in 1). This organism also contains GlcNAc-α-1-P in phosphodiester linkage to Serine residues. This modification, which we call phosphoglycosylation, was first identified in a cysteine proteinase, Proteinase I, present in vegetative cells (2-4). GlcNAc-α-1-P specific antisera recognize this protein (5, 6) and others in vegetative and developing stages of *Dictyostelium*. We recently cloned two vegetative cysteine proteinases, CP4 and CP5 (7). Both have serine-rich domains with contiguous repeats of three different motifs: SSS, SGSG and SGSQ. Expressed CP4 reacts with a GlcNAc-α-1-P-specific antibody and probably has this modification in one or more of these motifs (7). Since Proteinase I is also rich in serine and glycine(unpublished results), this led us to use a peptide with serine-glycine repeats to develop a GlcNAc-α-1-P transferase assay. This activity was detected and

*Corresponding Author. FAX: (619) 450-2101.

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the product shown to react with GlcNAc-α-1-P specific antibodies. We can now use the assay and antibody to look for mutants that lack GlcNAc-α-1-P transferase.

MATERIALS AND METHODS

Radiolabels and peptides. UDP [6-^{3}H] GlcNAc 60Ci/mmole was purchased from American Radiolabeled Chemicals, St. Louis, MO. Octanoyl-Asn-Tyr-Thr-NH_2 was synthesized by Omni Biochem., San Diego, CA. Cyclic peptide octanoyl-Gly-Pen-Ser-Asp-Asp-Tyr-Ser-Gly-Ser-Gly-Cys was a generous gift of Dr. Mario Bourdon, California Institute of Biological Research, La Jolla, CA. Penicillamine (Pen) forms a disulfide bond with Cys to yield the cyclic peptide.

Cell lines. All experiments were done with *D. discoideum* axenic strain AX4. Cells were grown to mid-log phase (3-5 x 10^6 cells/ml) in HL-5 (8).

Chromatographic analyses. QAE-Sephadex and anion exchange HPLC were performed as described (9). Thin layer chromatography of the labeled products was done on cellulose plates developed in ethyl acetate/acetic acid/water 6:4:3 which separates a variety of phosphorylated sugars. GlcNAc-α-1-P standard was detected by molybdate-perchloric acid spray (10) or other standards by silver nitrate stain (11).

Immunological procedures. Affinity purified GlcNAc-α-1-P specific IgG antibodies were prepared as previously described (6) using a rabbit antiserum prepared against Proteinase I. Further work describing the antibody will be presented elsewhere. Dot blots were done on nitrocellulose using approximately 0.4 nmole of peptide. The blot was blocked with 5% powdered milk, then incubated with 1:1000 dilution of antibody. Alkaline phosphatase-conjugated antibody binding was visualized with BCIP and NBT (12).

GlcNAc-α-1-P transferase assay. Assay conditions were similar to those used for GlcNAc-α-1-P assay described previously using α-methyl mannoside as an acceptor (13,14). That enzyme is the first step in the synthesis of phosphorylated *N*-linked oligosaccharides in *Dictyostelium*. Membranes were prepared by sonication as described (13). The acceptor peptide (0.3mM) and 10μM UDP-[^{3}H]GlcNAc (80,000-100,000 cpm) were added to membranes and incubated at 22°C for 1 hr. Tubes were heated for 3 min at 100°C, diluted with 0.5 ml of water, and spun at 10,000 xg for 5 min. The supernate was loaded onto a reverse phase C-18 cartridge (Alltech) and washed with 15 ml of water. One additional ml of water was washed through the cartridge and saved. The glycosylated peptide was eluted with 5 ml of 100% MeOH. The radioactivity in the final water wash (100-300 cpm) was subtracted from the amount that eluted with MeOH (1,000-14,000 cpm for samples with peptide) to correct for variable or incomplete washing. All values were then corrected for incorporation into endogenous acceptors in the absence of added peptide acceptor. No more than 10-15% of reactants or products were consumed during the assay.

RESULTS

Identification of an GlcNAc-α-1-P transferase activity. Since the known protein acceptors in *Dictyostelium* are rich in serine and glycine, we used a synthetic cylic peptide (octanoyl-Gly-Pen-Ser-Tyr-Asp-Asp-Ser-Gly-Ser-Gly-Ser-Gly-Cys) (15) as a potential acceptor of GlcNAc-α-1-P from UDP-GlcNAc. Microsomal membranes were incubated with UDP[^{3}H]GlcNAc and labeled peptide was isolated by binding to a

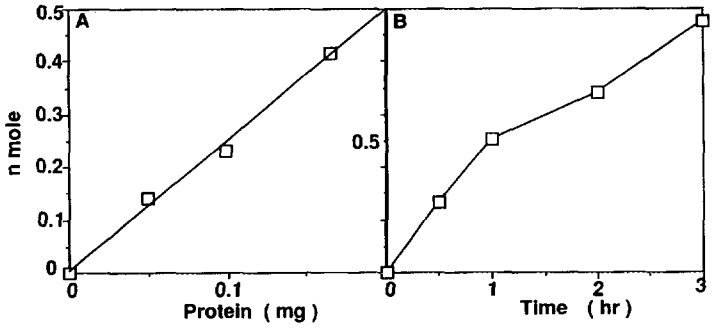

FIGURE 1. Effects of time and protein on the incorporation of ^{3}H-GlcNAc into synthetic peptide acceptor. Samples were incubated for 1 hr at 22°C with UDP [^{3}H] GlcNAc and various amounts of membrane protein (Panel A) or with 300 μg of protein for various times (Panel B).

C-18 cartridge. The reaction is linear with time and protein (Figure 1, Panels A and B). Since this peptide also contains a residue of Tyr in addition to Ser, it might also act as potential acceptor; however, octanoyl-Asn-Tyr-Thr-NH_2 does not serve as an acceptor (Figure 2) (16). The Km for the $(Ser\text{-}Gly)_3$ containing peptide acceptor was calculated at 275 μM with a Vmax of 3.71 nm/hr/mg protein (Figure 2, Panel A and insert), and the Km for UDP-GlcNAc was estimated at 20.6 μM (Figure 2, Panel B, and insert).

Analysis of the product. The peptide product bound to QAE-Sephadex and was eluted with 400 mM NaCl (Figure 3, Panel A). Mild base treatment of the peptide (0.05 N NaOH, 22° for 3 hr) releases 80% of the radioactivity, which elutes in the position expected for a molecule with two negative charges (Panel B). This was confirmed by

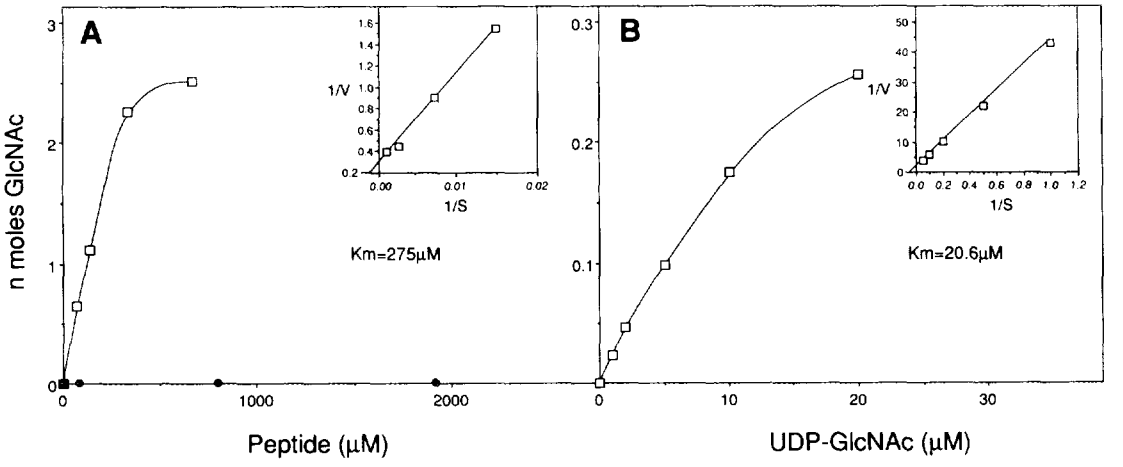

FIGURE 2. Effects of various concentrations of UDP-GlcNAc and peptide on glycosylation. Panel A 300 μg of membrane protein was incubated for 1 hr at 22°C with Ser-Gly (□-□) or Asn-Tyr-Thr (•—•) peptide acceptor. Similar incubations used 300 μM Ser-Gly-containing peptide and increasing amounts of UDP-GlcNAc (Panel B). Inserts show double inverse plots which were used to calculate Km values shown.

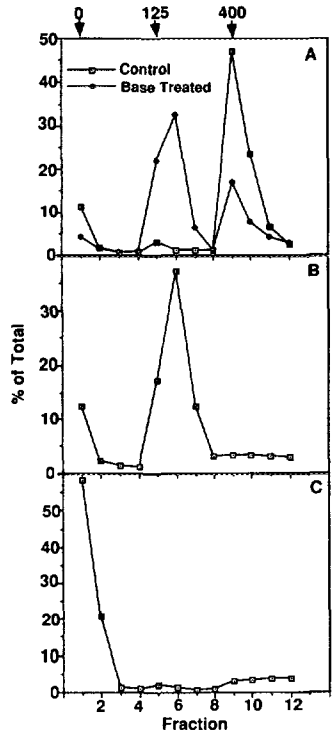

FIGURE 3. QAE-Sephadex analysis ^{3}H-labeled Ser-Gly-containing peptide. Purified labeled peptide from the C18 cartridge was run on QAE-Sephadex and eluted with water, 125 mM NaCl or 400 mM NaCl either before (▣—▣) or after (◆—◆) treatment with 0.1N NaOH for 4 hr (Panel A). The base treated sample was reapplied to the C18 column and non-binding material was analyzed on QAE-Sephadex (Panel B) or was treated overnight with *E. coli* alkaline phosphatase prior to QAE-Sephadex (Panel C).

anion-exchange HPLC (data not shown). The product co-migrates with authentic GlcNAc-α-1-P (Figure 4). Alkaline phosphatase digestion of this base-released material generates a neutral molecule (Figure 3, Panel C) that co-migrates with GlcNAc in thin layer chromatography (Figure 4). Very mild acid treatment (0.01N HCl, 95° C) of the peptide product releases all of the radioactivity as [^{3}H]GlcNAc (Figure 4) with a T1/2 of 5 min (not shown), which is typical for cleavage of phosphodiesters (13). Together these results show that the enzyme transferred GlcNAc-α-1-P to the peptide. The acceptor elutes as a single symmetrical peak from QAE-Sephadex and probably contains a single residue of GlcNAc-α-1-P per peptide (data not shown).

Reaction with GlcNAc-α-1-P specific antibodies. Dot blots with GlcNAc-α-1-P-specific antibodies recognize the peptide product, but not the unglycosylated peptide, and base treatment as above substantially reduces the intensity of the reaction. All of the antibody binding is competed by UDP-GlcNAc (Figure 5). These results show that the antibody binding epitope can be created in an *in vitro* assay.

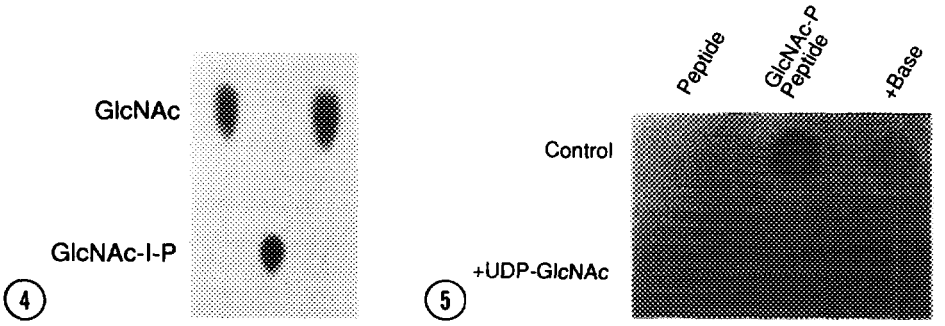

FIGURE 4. Characterization of the products formed from UDP-[^{3}H]GlcNAc in the presence of acceptor Ser-Gly-containing peptide. ^{3}H-labeled products released from labeled peptide were run on TLC plates. Authentic non-labeled standards were run in adjacent lanes for comparison. Left lane: peptide hydrolyzed in 0.01 N HCl for 10 min at 95°C; Center lane: base treated material (Figure 3) that eluted in 125 mM NaCl fraction after desalting on Dowex 50; Right lane: alkaline phosphatase treated sample from the center lane.

FIGURE 5. Dot-blot analysis of GlcNAc-α-1-P containing peptide, 0.4 n moles of peptide, phospho-*N*-acetylglucosaminylated peptide, or base treated (0.1 N NaOH, 22°C, 1 hr) phospho-*N*-acetylglucosaminylated peptide was blotted on nitrocellulose membrane filters and stained with the GlcNAc-α-1-P specific antibodies either in the absence (top row) or the presence of 5 mM UDP-GlcNAc.

DISCUSSION

We have shown here that *Dictyostelium* contains a membrane-bound activity that transfers GlcNAc-α-1-P from UDP-GlcNAc to a serine-containing acceptor peptide. Another peptide, Octanoyl-Asn-Tyr-Thr-NH_2 which will accept *N*-linked oligosaccharides from a lipid-linked donor is not an acceptor in this assay, even though it has two other hydroxy amino acids. This suggests that the GlcNAc-α-1-P reaction is preferential, and perhaps exclusive for Ser residues. The Km calculated for the Ser-Gly peptide is comparable to that seen when this peptide is used as an acceptor in a Xylosyl transferase assay, the initiatial step in mammalian proteoglycan synthesis (16). This does not necessarily mean that this peptide is the best or the only acceptor for the *Dictyostelium* GlcNAc-α-1-P transferase. The peptide was chosen based on its availability and the presence of three contiguous Ser-Gly repeats. A cluster of such repeats is found in a cloned cysteine proteinase of *Dictyostelium* that carries GlcNAc-α-1-P (7). Previous studies have suggested that most of the GlcNAc-α-1-P residues occur in a cluster on Proteinase I (2-4). Clearly, more refined characterization of the transferase and its range of potential acceptors are required along with localization of GlcNAc-α-1-P residues on the native protein. These studies are in progress.

Since *Dictyostelium* is haploid and amenable to mutagenesis (17), these results should provide a basis to screen for mutants that do not bind antibody and also lack GlcNAc-α-1-P transferase activity. Using the newly developed REMI technology

in *Dictyostelium* (18,19) may provide a way of isolating the GlcNAc-α-1-P transferase gene.

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