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Dictyostelium gnt15 encodes a protein with similarity to LARGE and plays an essential role in development

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Abstract

LARGE is a putative glycosyltransferase found to be mutated in mice with myodystrophy or patients with congenital muscular dystrophy. By homology searches, we identified in the *Dictyostelium discoideum* genome four open reading frames, i.e. *gnt12–15*, encoding proteins with sequence similarity to LARGE. Semi-quantitative RT-PCR analysis revealed distinct temporal expression patterns of the four *gnt* genes throughout *Dictyostelium* development. To explore the gene function, we performed targeted disruptions of *gnt14* and *gnt15*. The *gnt14*$^-$ strains showed no obvious phenotypes. However, *gnt15*$^-$ cells grew slowly, changed in morphology, and displayed a developmental phenotype arresting at early stages. Compared with the wild type, *gnt15*$^-$ cells were more adhesive and exhibited altered levels of some surface adhesion molecules. Moreover, lectin-binding analysis demonstrated that *gnt15* disruption affected profiles of membrane glycoproteins. Taken together, our data suggest that Gnt15 is essential for *Dictyostelium* development and may have a role in modulating cell adhesion and glycosylation.
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Glycosylation is a ubiquitous form of post-translational modification that profoundly affects expression and function of many proteins. Processing of oligosaccharides in cells requires the participation of many different enzymes, including glycosyltransferases that add sugar moieties to nascent polypeptides, and glycosidases that remove sugar moieties from glycoproteins [1]. It is noted that protein glycosylation changes during differentiation and development, under different physiological conditions, and in various diseases [2,3], indicating that glycosylation enzymes are highly regulated. An increasing number of genes encoding for putative or demonstrated glycosyltransferases have been associated with different forms of congenital muscular dystrophies which exhibit defective processing of α-dystroglycan (α-DG) [4,5]. Among these is *LARGE* [6] which encodes a predicted transmembrane protein containing two putative catalytic domains with the conserved DXD (Asp-any-Asp) motif typical of many glycosyltransferases [7]. The proximal catalytic domain of LARGE is most homologous to a bacterial glycosyltransferase family 8 (GT8) member [8] while the distal domain most resembles the human UDP-GlcNAc:Gal-β1,3-*N*-acetylglucosaminyltransferase (iGnT) [9], a member of GT49 family [8]. LARGE is required for the generation of functional, properly glycosylated forms of α-DG [10,11]. Recently a highly homologous gene *LARGE2* has been found and its product also supports the maturation of α-DG [12]. Despite the sequence feature and evidence suggesting their function in protein glycosylation, LARGE proteins have not been directly demonstrated to be bona fide glycosyltransferases.

To find targets for future comparative structure-function studies, we searched in lower eukaryotic model organisms for LARGE-like sequences. Here we report our discovery in *Dictyostelium discoideum*, an organism that undergoes a starvation-induced developmental program in which many glycoproteins serve essential functions [13]. We found four putative glycosyltransferase genes

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(*gnt12–15*) encoding products with significant similarity to mammalian LARGE proteins. Our initial characterization of *gnt14–15* revealed that *gnt15* (but not *gnt14*) is essential for development. We provide evidence for the involvement of Gnt15 in regulating growth, morphology, and adhesion of cells. Our results are also consistent with a role of Gnt15 in modulating glycosylation.

Materials and methods

Dictyostelium growth, development, and transformation. *Dictyostelium* cells were grown at 22 °C on SM plates with *Klebsiella aerogenes* or in HL5 medium [14]. For development, cells were washed and plated onto DB plates at 1.5×10^6 cells/cm^2 or developed in DB buffer with gentle shaking as described [14,15]. Transformation of cells with DNA was performed by electroporation [16]. Transformants were grown in HL5 with appropriate selection, i.e. 5 μg/ml blasticidin S (Cayla, France) or 10 μg/ml G418 (Sigma).

RT-PCR analysis of gene expression. Total RNA from wild-type (Ax2) cells at different developmental stages was isolated using Trizol (Gibco-BRL) according to the manufacturer's protocol. First strand cDNA was prepared by reverse transcription using an oligo-dT primer, and gene-specific primer sets (Supplementary Table S1) were used in subsequent PCR amplifications.

Plasmids and construction of *gnt14*$^-$ and *gnt15*$^-$ strains. See Supplementary materials and methods.

Spore assay. Cells developed on DB agar for 48 h were collected and assayed for spore formation as described [17]. Detergent-resistant spores were counted under microscopes and plated clonally on SM plates with bacteria to estimate the number of viable spores in each sample.

Adhesion assay. Cell–cell adhesion was assayed as described [18] with modifications. Cells were harvested, washed twice, and resuspended in PB (50 mM KH_2PO_4, 50 mM Na_2HPO_4 pH 6.1) with or without 10 mM EDTA to 5×10^6 cells/ml. After complete disperse of cells by vortexing, 1-ml aliquots of cell suspension were placed in 10-ml beakers and gently shaken at 100 rpm on a gyratory shaker for 20 min. Triplicate samples were taken and cells not in "clumps" (i.e. $\geqslant 3$ cells aggregated) were counted on a hemocytometer and % Adhesion (i.e. [total numbers of cells − numbers of cells not in clumps] ÷ total numbers of cells) was determined.

Western and lectin blot analyses on membrane proteins. Cells were harvested and washed twice in 20 mM sodium phosphate buffer, pH 6.5, resuspended to 1×10^8 cells/ml, and lysed by passing through 5 μm Nucleopore filters (Whatman). Crude extracts were centrifuged at 20000*g* for 30 m in at 4 °C. Pellets (i.e. membrane fractions) were dissolved in 1× sample buffer. Samples of equal protein amount were separated using 10% SDS–PAGE and transferred onto PVDF membranes (Millipore). For Western analysis, blots were blocked in 5% bovine serum albumin (BSA) for 1 h, incubated first with primary antibodies in TBS buffer containing 0.1% Tween 20 (TBST) at 4 °C overnight and subsequently with horse raddish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins (1:5000 in TBST) for 1 h, and detected by enhanced chemiluminescence (ECL). Primary antibodies used include anti-gp24 (1:2000), anti-gp80 (1:200), and anti-gp150 (1:2000) kindly provided by Dr. C.-H. Siu, at University of Toronto, and anti-gp130 (10 μg/ml) kindly provided by Dr. Catherine Chia, at University of Nebraska-Lincoln). For lectin-blot analysis, membranes were blocked in 5% BSA for 1 h, incubated first with 10 μg/ml biotin-lectins (including concanavalin A, winged bean agglutinin, and wheat germ agglutinin; in PBS buffer containing 1% BSA and 0.1% Tween 20) for 1 h, and subsequently with streptavidin-HRP (1:2000 in PBST) for 1 h, and detected by ECL.

Results and discussion

Dictyostelium gnt12–15 encode LARGE-like proteins

BLASTp searches in the *D. discoideum* genome database, i.e. the dictyBase (http://dictybase.org) [19] using human LARGE protein sequence as the query found four *Dictyostelium* open reading frames with sequence similarity to LARGE, i.e. *gnt14*, *gnt15*, *gnt13*, and *gnt12* in descending order of scores (Table 1). The four sequences are annotated as putative glycosyltransferases, possibly *N*-acetylglucosaminyltransferases (GlcNAc Transferases), hence named *gnt* genes.

We examined the sequences for shared motifs or domains. MEME (Multiple Expectation-maximization for Motif Elicitation, version 3.5.4; San Diego Supercomputer Center, UCSD; http://meme.sdsc.edu/meme) analysis identified conserved sequence blocks I–IV among all analyzed proteins; these blocks cluster in the C-terminal part of LARGE proteins (Supplementary Fig. S1). Gnt12–15 each possesses a DXD motif in block II, and ClustalW analysis revealed that these *Dictyostelium* DXD motifs are most similar to the third DXD motif in LARGE proteins (data not shown). All the analyzed proteins contain a predicted transmembrane domain (Fig. 1A), consistent with the possibility that they are ER- or Golgi-residing glycosyltransferases. Mammalian LARGE proteins contain two putative catalytic domains, the N-terminal GT8 and the C-terminal GT49 domains [8]. Only the GT49 domain is shared by *Dictyostelium* Gnt12–15; the degrees of similarity calculated over this region ranged from 44% to 59% (Table 1). Consistently, in a report describing results of a genome-wide survey of *Dictyostelium* sequences similar to glycosyltransferases classified in the CAZy (Carbohydrate-Active enZymes) database (http://afmb.cnrs-mrs.fr/CAZY), Gnt12–15 were also listed as GT49 β3GlcNAcT-like sequences [20]. Among Gnt12–15, only Gnt14 has the DXD motif located in the GT49-like region; Pfam HMM searches (The Sanger Institute; http://www.sanger.ac.uk/Software/Pfam/search.shtml) found that Gnt13 and Gnt15 have their DXD located in a region weakly matching the GT2 domain [8].

Dictyostelium gnt12–15 exhibit distinct temporal expression patterns during development

To determine if *Dictyostelium* indeed express *gnt12–15*, we performed RT-PCR analysis on RNA samples prepared from wild-type cells developed to different stages. Distinct temporal expression patterns throughout development were noted (Fig. 1B). Levels of *gnt14* and *gnt13* expression were relatively steady during vegetative growth and development. Conversely, *gnt15* showed highest expression at vegetative and pre-aggregation stages while *gnt12* expression peaked at mid- to late-stages of development. These results suggest that Gnt12–15 may function at distinct developmental stages.

Disruption of gnt15 but not gnt14 affects growth, morphology, and development

To explore their function, we further characterized the two *Dictyostelium* genes with highest LARGE-BLASTp

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