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A developmentally regulated gene product from *Dictyostelium discoideum* shows high homology to human α-L-fucosidase

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A cDNA library of poly(A$^+$)-RNA has been prepared from membrane-bound polysomes of *Dictyostelium discoideum* and screened for clones hybridizing to mRNA species that encode developmentally regulated proteins. The clone investigated in this paper recognizes a 1.8 kb transcript that accumulates strongly between the growth phase and aggregation stage. Stimulation of cells with pulses of cAMP enhances the accumulation. The amino acid sequence derived from a complete cDNA and from a genomic clone displays extensive sequence identity to human liver α-L-fucosidase. The *D. discoideum* DNA sequence encodes a 50.5 kDa polypeptide with a hydrophobic signal peptide at the N-terminus. Antibodies against a synthetic peptide corresponding to amino acids 262–275 of the deduced protein sequence recognize a developmentally regulated 50 kDa protein in *D. discoideum* that is recovered in the particulate fraction.

Fucosidase; Development; *(Dictyostelium)*

1. INTRODUCTION

During development from the growth phase to the aggregation stage, cells of *Dictyostelium discoideum* begin to communicate with each other by cAMP signals and by cell-to-cell contact. Both mechanisms of intercellular communication are based on the expression of new membrane proteins at the time of transition from the single cell to multicellular stage. The functions of only a few of these proteins are known. Among them are cAMP receptors [1] and a cell-adhesion molecule, the contact site A glycoprotein [2]. A number of cDNA clones represent still unidentified developmentally regulated proteins that are absent from growth-phase cells and present in aggregating cells. The expression of most of these mRNA species is enhanced by periodic cAMP signals, which are produced by *D. discoideum* cells before and during the aggregation stage [3,4]. Sometimes the function of the corresponding protein can be deduced from the cDNA-derived amino acid sequence, as was the case for a cysteine proteinase [5] and a serine esterase [6]. Here we report on a cAMP-controlled mRNA which codes for a protein with a high degree of homology to human α-L-fucosidase [7], and discuss the possible involvement of a fucosidase in the modification of protein- or lipid-linked carbohydrates in aggregation or later development of *D. discoideum*.

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The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession no.Y07497

2. MATERIALS AND METHODS

2.1. *Culture of* D. discoideum

Strain AX2-214 was grown axenically at 21°C. Cells were harvested at the exponential growth phase and washed free of nutrients by centrifugation in cold 17 mM Soerensen's buffer (Na_2HPO_4/KH_2PO_4), pH 6.0. For development cells were either dispensed onto nitrocellulose filters type HABG (Millipore) or kept in suspension on a rotary shaker at 150 rpm. Cells of strain AX3 (Kessin) were grown axenically at 23°C and starved in suspension as described. For stimulation of development, cAMP pulses of 20 nM amplitude were applied every 6 min to starving AX3 cells.

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2.2. *cDNA and genomic cloning*

The A11H2 clone was obtained by differential screening from a cDNA library of poly(A)$^+$-RNA isolated from membrane-bound polysomes of AX2 cells starved in suspension for 6 h [8,9]. A 0.96 kb *Eco*RI fragment of this partial clone was nick-translated with (α-^{32}P)-labeled dATP [10] and used for library screens, Northern and Southern blots. The probe represented the 3'-region downstream of the internal *Eco*RI site (fig.1). Plasmid DNA was isolated according to Birnboim and Doly [11]. cDNA clone cDF2 was obtained by screening a λgt11 cDNA library provided by Dr R. Kessin (Columbia University, NY). The library was made from RNA isolated from AX3 cells that had been induced by cAMP [12]. Recombinant phages were grown on *E. coli* strain Y1088 [13] for the isolation of DNA. The genomic clone gDF6 was obtained by screening an EMBL3 library prepared from a partial *Sau*3A digest of genomic AX2 DNA [14]. Recombinant EMBL3 phages were propagated on *E. coli* LE392 [15].

2.3. *Sequence analysis*

Restriction cleavage maps and the sequencing strategy are shown in fig.1. The inserts were subcloned into vectors M13mp18/19 or pUC18/19 [16]. Deletion clones of the pUC subclones were produced by exonuclease III/exonuclease S_1

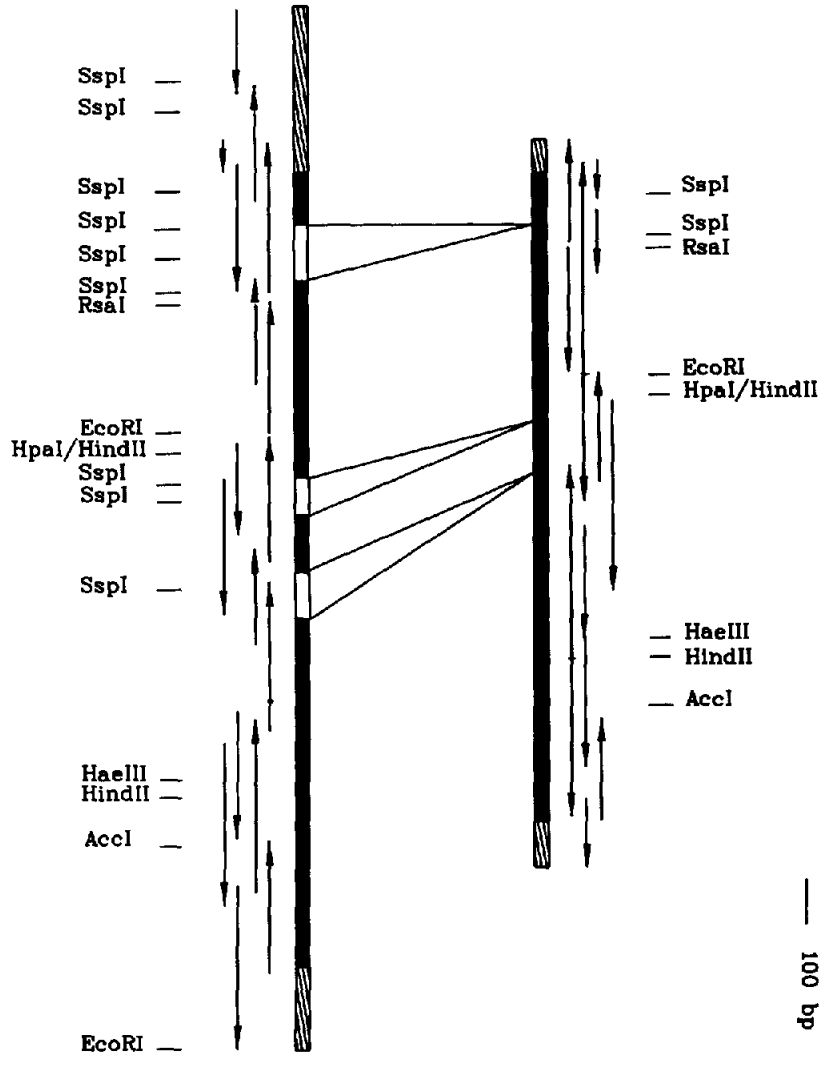

Fig.1. Sequencing strategy of cDNA and genomic clones of the A11H2 gene. Cleavage sites for restriction enzymes and directions of nucleotide sequencing of the complete cDNA clone cDF2 (top) and the genomic clone gDF6 (bottom) are indicated. Coding sequences are depicted by solid bars, introns by open bars, and 5'- and 3'-noncoding sequences by hatched bars. Nucleotide sequences were determined using sequence-specific oligonucleotide primers or subclones.

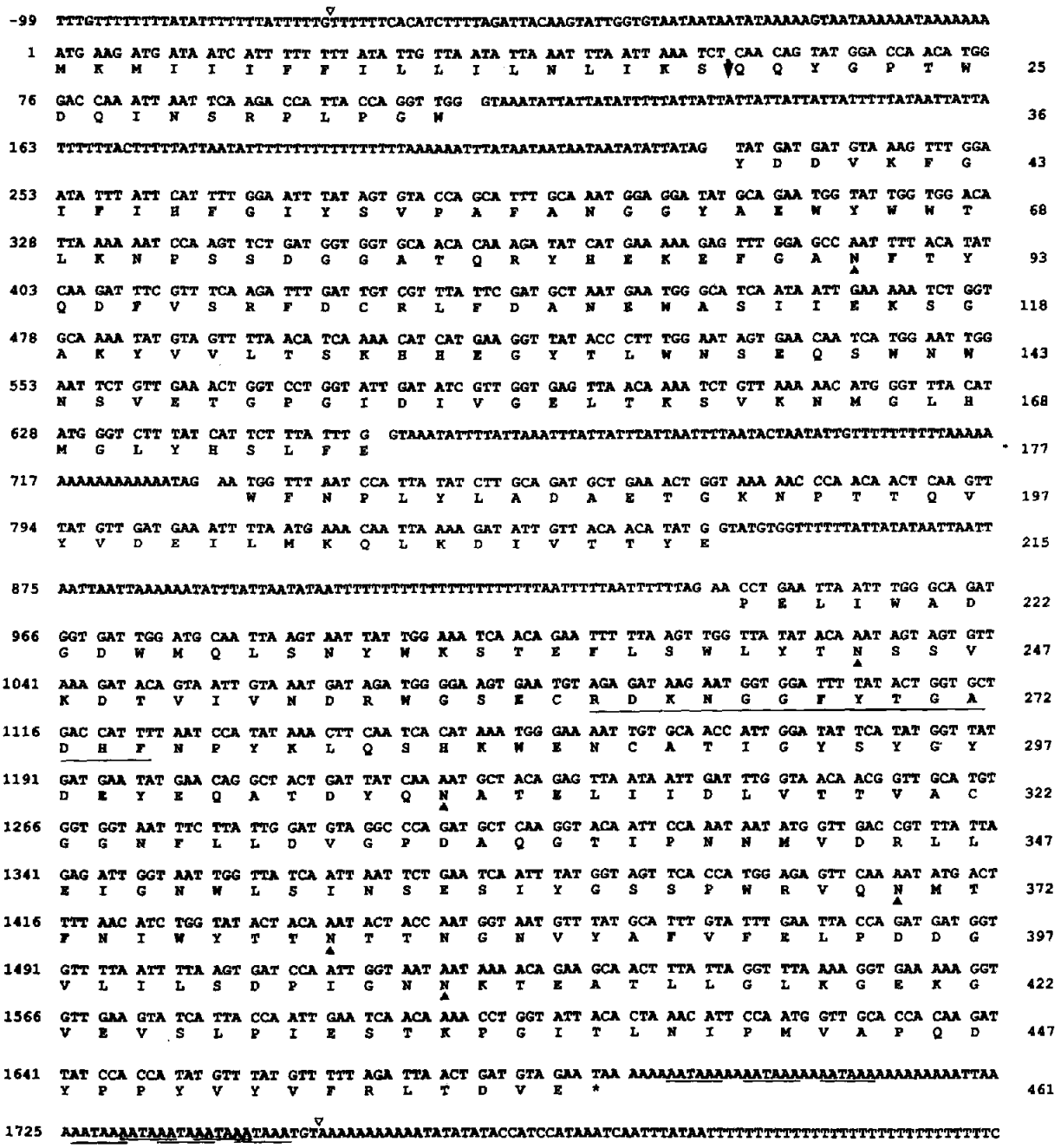

Fig.2. Nucleotide and deduced amino acid sequence of the genomic clone gDF6. In the 3′-noncoding region 8 possible polyadenylation signals are underlined. An arrow marks the putative cleavage site of the signal peptidase [24]. Consensus sequences for *N*-linked glycosylation are depicted by filled triangles. The underlined sequence was used for the preparation of a synthetic peptide. The start and the end of the cDNA clone cDF2 are indicated by unfilled, inverted triangles. The cDF2 sequence is identical with that of gDF6, apart from lacking the three introns.

treatment (Promega Biotec). For sequencing the dideoxy method [17] was applied using uni, reverse, or 18-mer oligonucleotide primers. Nucleotide and deduced amino acid sequences were analysed with the University of Wisconsin Genetics Computer Group (UWGCG) and the Protein Identification Resource (PIR) computer programs.

2.4. *DNA and RNA isolation and hybridization*

Total cellular RNA isolated from strain AX2 or AX3 cells was purified by several phenol/chloroform extractions. 10 μg RNA were applied per lane, separated by electrophoresis in 1.2% agarose gels in the presence of 6% formaldehyde [18], and transferred onto nitrocellulose filters (BA85, Schleicher & Schüll). Northern blots were hybridized with nick-translated probes at 37°C in 50% formamide, 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate), 4 × Denhardt's solution, 4 mM EDTA, 1% sarcosyl, 0.12 M sodium phosphate buffer, pH 6.8, and 0.1% SDS.

2.5. *Anti-peptide antibodies and immunoblotting*

A peptide corresponding to amino acids 262–275 (fig.2) was synthesized on a *p*-alkoxybenzyl alcohol resin as described [19]. The peptide was linked by a lysylglycylglycyl N-terminal spacer to a palmitic acid residue [20]. 200 μg of the palmitoyl peptide in 1 ml emulsion of complete Freund's adjuvant were injected subcutaneously into New Zealand White rabbits, followed by 3 injections with incomplete Freund's adjuvant.

Particulate fractions of *D. discoideum* AX2 were prepared from growth-phase and from 6 h developed suspension culture cells. The cells were washed twice with phosphate buffer, pH 6.0, and lysed by freeze-thawing. The lysates were centrifuged at 4°C for 1 h at 100000 × *g* and aliquots of the pellet corresponding to 1 × 10^6 cells were loaded per lane on 10% SDS-polyacrylamide gels [21]. Proteins were transferred to nitrocellulose [22], the filters incubated for 1 h in 1 × NCP-buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.02% NaN_3) containing 8% bovine serum albumin and baked for 30 min at 100°C. The blots were subsequently incubated in 1:500 diluted rabbit anti-peptide serum, washed in 1 × NCP-buffer, and labeled with ^{125}I-goat anti-rabbit IgG.

3. RESULTS

3.1. *The amino acid sequence deduced from the* D. discoideum *A11H2 gene shows a high degree of homology to human α-L-fucosidase*

One cDNA and one genomic clone of the A11H2 gene were sequenced. Both clones comprised the complete coding region. Since no differences were found between the sequences of the cDNA and the exons of the genomic clone, only the sequence of the genomic clone is shown (fig.2). This clone contained three AT-rich introns with lengths of 79, 97 and 123 base pairs. In the 3'-flanking region the sequence showed 8 possible polyadenylation signals. The cDNA clone consisted of 1522 bases, plus a poly(A) tail of 27 nucleotides starting at position 1752 in fig.2. The codon usage is comparable to that of other genes of *D. discoideum* [23].

The deduced amino acid sequence indicates a primary translation product of 461 residues including a hydrophobic N-terminal leader sequence. On the basis of consensus sequences for signal peptidases [24], the leader is assumed to consist of 18 amino acids, the processed protein starting with glutamine 19 of the sequence shown in fig.2. The calculated molecular mass of the putative mature protein is then 50.5 kDa. The sequence reveals the presence of 6 potential *N*-glycosylation sites, five being in the C-terminal half of the molecule. The protein is characterized by an unusually high tryptophan content of 19 residues, which corresponds to 4.3 mol%.

Computer searches at the amino acid level revealed strong similarity with only one other known sequence, that of human α-L-fucosidase. The sequence of this polypeptide has been obtained from an incomplete cDNA clone isolated from a hepatoma expression library by screening with anti-α-L-fucosidase antibodies [7]. The homology between the *D. discoideum* A11H2 sequence and the partial human sequence extends over the entire length of the latter (fig.3). Within the shared region, 46% of the amino acids are identical. In addition, there are a number of conservative amino acid exchanges. The human clone represents 80% of the mature α-L-fucosidase. The *D. discoideum* sequence comprises, not regarding the leader, 44 additional amino acids in the N-terminal region and 44 in the C-terminal one.

The cDNA-derived human and *D. discoideum* sequences were also compared in hydropathy plots. In general, the profiles are similar, but a hydrophobic stretch in positions 311–331 is more pronounced in the *D. discoideum* sequence than in the human one. Neither this stretch nor another hydrophobic region at positions 41–59, which is not represented in the human clone, has the characteristics of a transmembrane domain [25].

Southern blots of genomic *D. discoideum* DNA were probed with a cDNA fragment that comprises the 3'-portion of the coding region starting from the internal *Eco*RI site (fig.1), and extends into 3'-flanking sequences. When using restriction enzymes that do not cleave within the A11H2 gene, for instance *Hpa*II, only one fragment was

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A11H2   MKMIIIFFILLILNLIKSQQYGPTWDQINSRPLPGWYDDVKFGIFIHFGIYSVPAFANGG   60

A11H2   YAEWYWWTLKNPSSDGGATQRYHEKEFGANFTYQDFVSRFDCRLFDANEWASIIEKSGAK  120
α-Fuc     EFLWWHWQGEGRP--QYQRFMRDNYPPGFSYADFGPQFTARFFHPEEWADLFQAAGAK   56

A11H2   YVVLTSKHHEGYTLWNSEQSWNWNSVETGPGIDIVGELTKSVKNMGLHMGLYHSLFEWFN  180
α-Fuc   YVVLTTKHHEGFTNWPSPVSWNWNSKDVGPHRDLVGELGTALRKRNIRYGLYHSLLEWFH  116

A11H2   PLYLADAETGKNPTTQVYVDEILMKQLKDIVTTYEPELIWADGDWMQLSNYWKSTEFLSW  240
α-Fuc   PLYLLDKKNGFK--TQHFVSAKTMPELYDLVNSYKPDLIWSDGEWECPDTYWNSTNFLSW  174

A11H2   LYTNSSVKDTVIVNDRWGSECRDKNGGFYTGADHFNPYKLQSHKWENCATIG-YSYGYDE  299
α-Fuc   LYNDSPVKDEVVVNDRWGQNCSCHHGGYYNCEDKFKPQSLPDHKWEMCTSIDKFSWGYRR  234

A11H2   YEQATDYQNATELIIDLVTTVACGGNFLLDVGPDAQGTIPNNMVDRLLEIGNWLSINSES  359
α-Fuc   DMALSDVTEESEIISELVQTVSLGGNYLLNIGPTKDGLIVPIFQERLLAVGKWLSINGEA  294

A11H2   IYGSSPWRVQ--NMTFNIWYTTNTTNGNVYAFVFELPDDGVLILSDPIGNNKTEATLLGL  417
α-Fuc   IYASKPWRVQWEKNTTSVWYTSKGS--AVYAIFLHWPENGVLNLESPITTSTTKITMLGI  352

A11H2   KGEKGVEVSLPIESTKPGITLNIPMVAPQDYPPYVYVFRLTDVE                  461
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Fig.3. Alignment of amino acid sequences of the *D. discoideum* A11H2 gene product and the incomplete cDNA-derived sequence of human α-L-fucosidase [7]. Identical amino acids are indicated by vertical bars, conservative exchanges by dots. Conservative exchanges are based on the PIR permutation matrix and grouped as D, E, Q, N; F, Y, W; I, L, V, M; S, T, A, G; and H, R, K. Five gaps of 1 or 2 amino acids are indicated by dashes.

recognized. All the other fragment patterns obtained with different restriction enzymes were consistent with the genome of *D. discoideum* containing only a single copy of the A11H2 gene.

3.2. *Transcription of the A11H2 gene is developmentally regulated and controlled by cAMP*

Growth-phase cells of *D. discoideum* AX2 were transferred onto nitrocellulose filters for development and reached the aggregation stage within 12 h (fig.4). RNA isolated at intervals during development showed only a single A11H2 transcript of 1.8 kb. This transcript accumulated from undetectable amounts in growth-phase cells to a maximum degree of expression in aggregating cells. The amount of A11H2 mRNA slowly declined during the 6 h period following aggregation, which corresponded to the tipped aggregate and slug stages and was no longer detected at the 21 h stage of culmination. For comparison, mRNA for the contact site A protein was labeled. This strictly regulated mRNA is known to be expressed shortly before the onset of aggregation, and to decay later during development [2]. The amount of contact site A mRNA peaked at 12 h of development, at the same time as the A11H2 mRNA (fig.4).

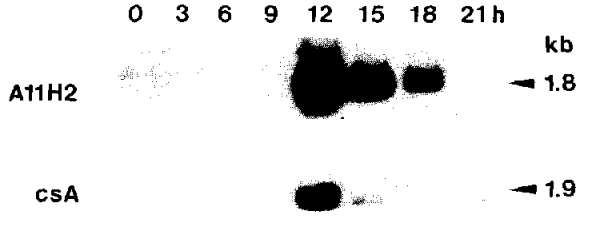

Fig.4. Developmental regulation of *D. discoideum* A11H2 mRNA. Total RNA from different stages of development as indicated in hours, was probed with nick-translated cDNA fragments specific for either A11H2 or contact site A transcripts (csA). The 12 h stage of development on nitrocellulose filters corresponded to aggregation, the 18 h stage to slug formation. RNA sizes in kb are indicated.

The effect of cAMP pulses on the accumulation of A11H2 transcripts was investigated in suspension cultures of AX3 cells, which respond strongly to pulsatile addition of cAMP. As in AX2, no transcript was detectable in axenically growing AX3 cells. With no cAMP applied, a transient weak expression was observed at 2 h of development, only trace amounts of transcript were detected at 4 h, and a moderate accumulation was seen at 6 h (fig.5). In cells stimulated by cAMP pulses, strong accumulation at 4 h, and a further increase at 6 h of development were observed, indicating that cAMP signals are involved in the accumulation of A11H2 mRNA.

3.3. *A developmentally regulated 50 kDa protein is labeled by anti-peptide antibodies*

To obtain a probe for the A11H2 protein, a rabbit antiserum was raised against a peptide corresponding to amino acids 262–275 (fig.2). The antibodies were applied to protein blots from particulate fractions of growth-phase and aggregation-competent *D. discoideum* cells (fig.6). A 19 kDa protein was detected in both fractions, while a 50 kDa protein was expressed only during the preaggregation phase. The developmental regulation of this protein parallels that of the A11H2 mRNA, and the apparent molecular mass of the protein is consistent with the sequencing data, provided that any carbohydrate moieties that are present do not substantially contribute to the electrophoretic mobility. The nature of the 19 kDa band is unknown.

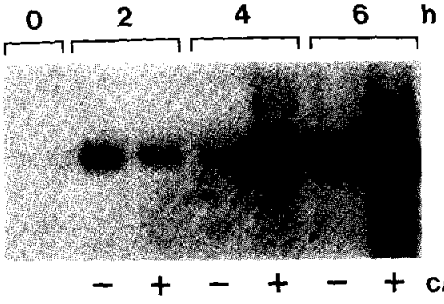

Fig.5. Effect of cAMP pulses on the accumulation of A11H2 mRNA. Total RNA was isolated from AX3 cells immediately after harvesting from nutrient medium (0 h) or after different times of starvation in suspension. One aliquot of cells was stimulated by pulses of cAMP during development (+), the other aliquot remaining unstimulated (–). The blot was probed with a nick-translated cDNA fragment specific for A11H2.

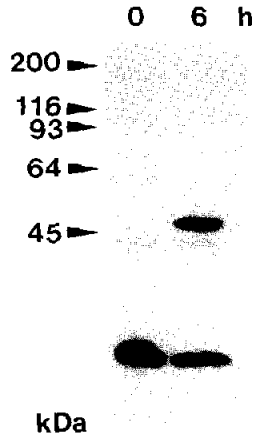

Fig.6. Proteins labeled in membrane fractions by anti-peptide antibodies. AX2 cells were harvested either from nutrient medium (0 h) or starved in suspension to acquire aggregation competence (6 h). The rabbit antibodies were raised against a synthetic peptide corresponding to the portion of the A11H2 sequence underlined in fig.2. Positions of molecular mass markers are indicated.

4. DISCUSSION

The developmentally regulated A11H2 gene of *D. discoideum* codes for a protein which is highly homologous to human α-L-fucosidase (EC 3.2.1.51). This homology is not restricted to a certain region, as one would expect if only the catalytic site is conserved, but is distributed throughout the entire length of the available human cDNA sequence [7]. The *D. discoideum* cDNA and genomic clones comprise the entire coding region including a hydrophobic leader. The calculated molecular mass of the mature *D. discoideum* polypeptide is 50.5 kDa. This is consistent with the apparent molecular mass reported for the subunits of the human liver enzyme [26] as well as with the apparent molecular mass of a *D. discoideum* protein determined by immunolabeling under denaturing conditions (fig.6). The human α-fucosidase has been reported to consist of two distinct subunits, one of 51 kDa, the other of 56 kDa [26]. The *D. discoideum* A11H2 sequence may correspond to only one of the subunits of the human enzyme.

The A11H2 protein seems to be controlled by the same cAMP-receptor mediated regulatory

mechanisms as a number of other proteins that are maximally expressed in aggregating cells, for instance the contact site A cell adhesion protein [8,9] and cell-surface cAMP receptors [1,27]. Promoter elements responsible for the cAMP-dependent regulation of the A11H2 gene have been identified (May, T. et al., in preparation).

Although direct proof that the A11H2 gene product is a fucosidase is not available as yet, the overall, strong homology with human α-L-fucosidase makes it worthwhile to discuss the possible functions of a fucosidase in *D. discoideum*. It is unlikely that these functions are associated with nutrition and propagation of *D. discoideum* cells, since the protein is not present during growth. This is consistent with previous results indicating that *D. discoideum* cells growing on bacteria degrade the lipid moiety of various bacterial lipopolysaccharides but not their carbohydrate residues [28,29]. It is likely that glycoconjugates produced by *D. discoideum* cells are substrates for fucosidases. For example, fucosylated glycosphingolipids of *D. discoideum* cells [30] are candidate substrates for endogenous fucosidases. This possibility is of particular interest, since lack of the α-L-fucosidase results in a lipid storage disease in man [31]. In addition, a number of fucosylated glycoproteins are expressed during development [32], including the contact site A glycoprotein. *N*-linked oligosaccharide residues have been implicated in the activity of this protein as a cell adhesion molecule [33], and an enzyme removing fucose residues might be involved in regulating this activity. Later in development, extensive incorporation of fucose precedes the differentiation of prespore cells, indicating the synthesis of fucosylated, cell-type specific glycoproteins at an early step of cell differentiation [34]. Again, a fucosidase may act in modifying the carbohydrate residues of these glycoproteins, probably during the recycling of plasma membrane proteins [35]. In *Polysphondylium pallidum*, an organism related to *D. discoideum*, a specific fucose-containing epitope, recognized by a monoclonal antibody, is controlled by genes that are also involved in the timing of development [36].

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