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Isolation and sequence analysis of a cDNA encoding rat liver α-L-fucosidase

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cDNA clones for α-L-fucosidase were isolated from a rat liver λgt11 expression library by using both monospecific polyclonal antibodies against the affinity-purified enzyme and biotinylated rat liver fucosidase cDNA sequences as probes. The largest clone, λFC9, contained a 1522 bp full-length cDNA insert (FC9) that encoded the 434-amino acid-residue subunit (M_r 50439) of rat liver α-L-fucosidase. A putative signal peptide 28 amino acid residues in length preceded the sequence for the mature protein. In addition, FC9 specified for 11 nucleotide residues of 5′ untranslated sequence, 78 nucleotide residues of 3′ untranslated sequence and a poly(A) tail. The deduced amino acid sequence from FC9 in conjunction with the experimentally determined *N*-terminus of the mature enzyme suggested that rat liver fucosidase did not contain a pro-segment. However, there was the possibility of limited *N*-terminal processing (one to five amino acid residues) having occurred after removal of the predicted signal peptide. Amino acid sequences deduced from FC9 were co-linear with amino acid sequences measured at the *N*-terminus of purified fucosidase and on two of its CNBr-cleavage peptides. An unusual aspect of rat liver α-L-fucosidase protein structure obtained from the FC9 data was its high content of tryptophan (6%). The coding sequence from FC9 showed 82% sequence identity with that from a previously reported incomplete human fucosidase sequence [O'Brien, Willems, Fukushima, de Wet, Darby, DiCioccio, Fowler & Shows, (1987) Enzyme **38**, 45–53].

INTRODUCTION

The catabolism of complex asparagine-linked glycoproteins in rat liver lysosomes occurs by way of an ordered bidirectional process [1]. Our laboratory is currently studying the biochemistry and molecular biology of the glycosidases involved in this glycoprotein degradation. In the overall degradative scheme lysosomal α-L-fucosidase (EC 3.2.1.51) is responsible for hydrolysing the α-1,6-linked fucose joined to the reducing-end *N*-acetylglucosamine of the carbohydrate moieties. The mature enzyme from rat liver has an approximate subunit M_r of 55000 and exists as a tetramer in the native state [2]. A deficiency of this acid hydrolase in humans and dogs results in the lysosomal storage disease fucosidosis [3–5].

The decision to initiate cloning studies with fucosidase in the rat was twofold. First, this lysosomal glycosidase is easily purified from rat liver by affinity chromatography [2], allowing its use as an antigen to prepare monospecific polyclonal antibodies. The purified enzyme could also be used to determine the partial amino acid sequence of rat liver fucosidase, thereby confirming the identity of isolated cDNAs. Secondly, there was available partial cDNA sequence information on human fucosidase with which to compare putative rat fucosidase gene sequences [6].

In the present paper we report the isolation of a full-length cDNA (FC9) encoding rat liver α-L-fucosidase from a rat liver λgt11 expression library. The nucleotide and deduced amino acid sequences of rat fucosidase clone FC9 are presented and compared with an incomplete human fucosidase sequence.

EXPERIMENTAL

Materials

Restriction endonucleases and T4 ligase were obtained from International Biotechnologies (New Haven, CT, U.S.A.) Sequenase DNA sequencing kit was purchased from United States Biochemical Corp. (Cleveland, OH, U.S.A.) Random-primed-DNA-labelling kit was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.) M13 cloning vectors mp18 and mp19 were from Pharmacia (Piscataway, NJ, U.S.A.). Goat anti-(rabbit IgG Fc) antibody–alkaline phosphatase conjugate, 5-bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium were obtained from Promega (Madison, WI, U.S.A.). Strepavidin–alkaline phosphatase conjugate was from Bethesda Research Laboratories (Bethesda, MD, U.S.A.). Nylon and nitrocellulose filters were purchased from Amersham–Searle (Arlington Heights, IL, U.S.A.). Immobilon [poly(vinylidene difluoride)] membranes were purchased from Millipore (Bedford, MA, U.S.A.). Biotin-11–dUTP was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [α-[^{35}S]thio]dATP was purchased from New England Nuclear–Dupont (Wilmington, DE, U.S.A.).

Immunological screening of λgt11 expression library

α-L-Fucosidase was affinity-purified from rat liver as described by Opheim & Touster [2]. Polyclonal antibodies raised against the purified enzyme were used to immunologically screen a λgt11 rat liver library (generously provided by Dr. C.-P. David Tu, The Pennsylvania State University) according to the method of Young & Davis [7]. The antigen–antibody complex was detected by using

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These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number X16145.

a goat anti-(rabbit IgG Fc) antibody–alkaline phosphatase conjugate followed by colour development with the substrates 5-bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium. Putative positive clones were taken through successive screening rounds at lower bacteriophage titres until homogeneous.

Identification of expressed fusion proteins

The purified bacteriophages from selected putative fucosidase clones were used to prepare lysogens of *Escherichia coli* Y1089 [8]. Protein lysates from temperature-induced liquid cultures of the lysogens were subject to SDS/polyacrylamide-gel electrophoresis according to the procedure of Laemmli [9]. The separated proteins were electrophoretically transferred [10] to an Immobilon membrane and either stained for protein with Amido Black or immunostained by using the same detection procedure as that used for immunoscreening.

Epitope selection

Antibodies to rat liver fucosidase were affinity-purified by use of the β-galactosidase fusion protein expressed by the λgt11 clone of interest [8]. The selected antibodies were used to probe immunoblots of purified and partially purified rat liver fucosidase preparations.

Subcloning and DNA sequencing

After *Eco*RI digestion of purified bacteriophage DNA from selected putative positive clones, released cDNA inserts were separated from the bacteriophage arms on a preparative agarose gel, excised from the gel and electro-eluted [11]. The isolated inserts were ligated into plasmid pUC18 to obtain preparative amounts of the insert and into cloning vectors M13mp18 and M13mp19 for sequence analysis. The nucleotide sequence was determined by the dideoxynucleotide chain-termination procedure [12] with the use of Sequenase [13].

Hybridization screening with biotin-labelled fucosidase cDNAs

The cDNA isolated from rat fucosidase clone λFC2 (FC2 800 bp) was random-primer-labelled with a biotinyl-dUTP analogue, biotin-11-yl-dUTP. Approx. 2×10^6 plaques of the λgt11 rat liver library were plated out on 150 mm² plates and screened with the biotin-labelled clone FC2 by using a modification of the procedure described by Kincaid & Nightingale [14]. Putative fucosidase clones were purified and their cDNA sizes were determined by restriction analyses. The largest of the eight clones detected, λFC24 (1163 bp), was digested with *Sal*I to generate a 5′ *Sal*I fragment 630 bp in length that extended 401 bp beyond the 5′ end of clone FC2. This 5′ *Sal*I fragment was random-primer-labelled with biotin-11-yl-dUTP and used as a probe to screen approx. 10^7 plaques of the rat liver cDNA library. All positive clones detected by the biotinylated probe were purified through successive screening rounds.

Amino acid sequence

Partial amino acid sequence information for purified rat liver α-L-fucosidase was experimentally determined from the *N*-terminus and from two CNBr-generated peptide fragments [15].

Computer-assisted sequence analysis

Searches for sequence similarities and sequence analyses were performed on a VAX (Digital Equipment Corp.) computer using software distributed by the Genetics Computer Group (University of Wisconsin). Nucleotide and protein sequences were also analysed by using the computer software DNA Strider [16].

RESULTS AND DISCUSSION

Isolation of cDNA clones for rat liver α-L-fucosidase

Approx. 10^6 plaques of the rat liver λgt11 expression library were screened with rabbit anti-fucosidase antibody, and seven putative positives were isolated and purified. *Eco*RI digestion of the purified bacteriophage DNA from the three most antigenic of these positives yielded an 800 bp insert from the clone designated λFC2, but no insert could be retrieved from the other two. The following three lines of evidence indicated that the insert from clone λFC2 contained sequences specific for rat liver fucosidase. (i) Lysogenic *E. coli* Y1089 harbouring the recombinant bacteriophage λFC2 expressed a fusion protein of M_r 143000 that was shown to react strongly with the anti-fucosidase antibody (Fig. 1). Taking into account the M_r of native β-galactosidase (116000), this suggested that the insert FC2 encoded a peptide with an M_r of approx. 27000, which is one-half the M_r of rat liver fucosidase as measured by SDS/polyacrylamide-gel electrophoresis [2]. Epitope selection experiments showed that the antibody fraction that was affinity-purified by the β-galactosidase–FC2 fusion protein was specific in its immunoreactivity towards rat liver fucosidase (results not shown). This specificity was not observed for antibodies purified with native β-galactos-

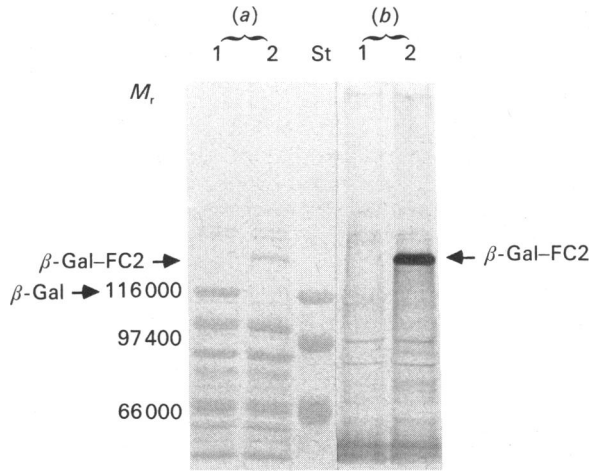

Fig. 1. Identification of a fucosidase clone from a λgt11 cDNA expression library by blot analysis of a fusion protein

Protein lysates from *E. coli* Y1089 infected with wild-type λgt11 (lane 1) or rat liver fucosidase clone λFC2 (lane 2) were separated by electrophoresis on a SDS/5% polyacrylamide gel and electrophoretically transferred to an Immobilon membrane. M_r standards (St) were also run. The left hand of the blot (*a*) was stained with Amido Black and the right half (*b*) was immunostained as described in the text. Arrows show the location of bands corresponding to native β-galactosidase (β-Gal) expressed by λgt11 and the β-galactosidase fusion protein (β-Gal–FC2), expressed by the recombinant clone λFC2.

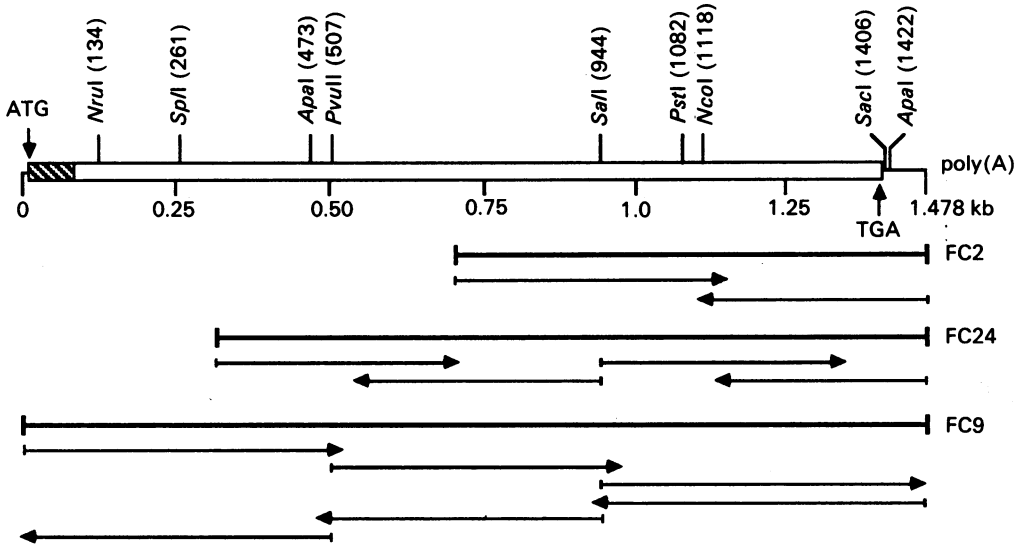

Fig. 2. Restriction map and sequencing strategy

The open box represents the protein coding sequence for mature rat liver fucosidase and the hatched box represents the coding sequence for the proposed signal peptide. The first in-frame translational start and stop codons are indicated by ATG and TGA respectively. Only the restriction sites used for sequencing and mapping are given. The sizes [excluding the poly(A) tail] of the three fucosidase cDNAs FC2, FC24 and FC9 and their relative positions are given below the restriction map. Horizontal arrows below each cDNA indicate the direction and extent of sequence determination.

idase. (ii) DNA sequence analysis of clone FC2 revealed an open reading frame of 680 bp that was punctuated with the stop codon TGA (Fig. 2). The open reading frame was shown to be similar to a partial human fucosidase sequence [6], having identical nucleotides at 80% of the comparable positions. The predicted amino acid sequences were 77% identical. (iii) A region of the deduced amino acid sequence from clone FC2 was found to be perfectly co-linear with a 16-amino acid-residue sequence obtained from a CNBr-cleavage peptide that was generated from rat liver fucosidase.

The rat liver λgt11 library was screened for additional clones by using the partial rat liver α-L-fucosidase cDNA FC2. Eight positives were isolated and plaque-purified after screening of approx. 2×10^6 plaques of the library. Restriction analyses of the bacteriophage DNA by use of sites identified within the sequence of FC2 verified the clones as being derived from the rat liver α-L-fucosidase gene. Although the DNA sequence of the longest of the eight clones, λFC24, indicated that its 5′-terminus extended approx. 400 bp beyond that of FC2 (Fig. 2), it did not contain the entire 5′-end of the coding sequence. In order to screen for a cDNA containing sequence from the 5′-end of the fucosidase gene, a 630 bp *SalI* fragment was generated from the 5′-end of FC24 and used to screen the rat liver λgt11 library. Restriction mapping of the 40 positives isolated from the 10^7 plaques that were screened confirmed their identities and revealed the existence of a potential full-length cDNA, FC9.

Sequence analysis of fucosidase cDNA clones

DNA sequencing of the three fucosidase clones FC2, FC24 and FC9 was done in accordance with the strategy outlined in Fig. 2. All three cDNAs were found to have their 3′-ends initiate in the poly(A) tail, and also to be identical in sequence in the areas that overlapped. Sequence analysis of the 1522 bp from FC9 suggested that this cDNA contained the entire coding region for the rat liver α-L-fucosidase gene. The complete nucleotide sequence and deduced amino acid sequence of rat fucosidase clone FC9 are compared in Fig. 3 with those compiled from three partial human fucosidase clones [6]. The deduced amino acid sequence from FC9 contained an in-frame methionine (ATG) 28 residues upstream to a string of 12 amino acid residues (Ala-29–Leu-40) that were exactly co-linear with the *N*-terminal sequence obtained from rat liver fucosidase (Fig. 3). Designating this first methionine residue +1 of the open reading frame, the coding region continued for 462 amino acid residues, where it terminated with a stop codon (TGA). The amino acid sequence between Met-1 and Ala-29 was found to have a predominance of hydrophobic residues characteristic of signal peptides [17,18]. Subtracting the relative molecular mass contributed by the proposed signal sequence, the deduced amino acid sequence from FC9 predicted the mature rat liver fucosidase polypeptide to have an M_r of 50439. In addition to the coding sequence, FC9 contained 11 nucleotide residues of 5′ untranslated sequence, 78 nucleotide residues of 3′ untranslated sequence and a 44 bp poly(A) tail. A polyadenylation signal (AATAAA) was noted in the 3′ untranslated region 14 nucleotide residues upstream from the poly(A) tail. Amino acid sequences from the two CNBr-cleavage peptides that were generated from purified rat liver fucosidase were shown to be perfectly co-linear with residues Lys-90–Gly-124 and Ser-307–Val-322 encoded by clone FC9.

Comparison of the rat and human sequences revealed 82% sequence identity at both the nucleotide and amino acid levels. All four potential glycosylation sites (Asn-237, Asn-247, Asn-264 and Asn-378) were found to be conserved between the two species. A major difference, however, between the two sequences was the lengths of their 3′ untranslated regions, 78 bp and 631 bp for the rat and human clones respectively.

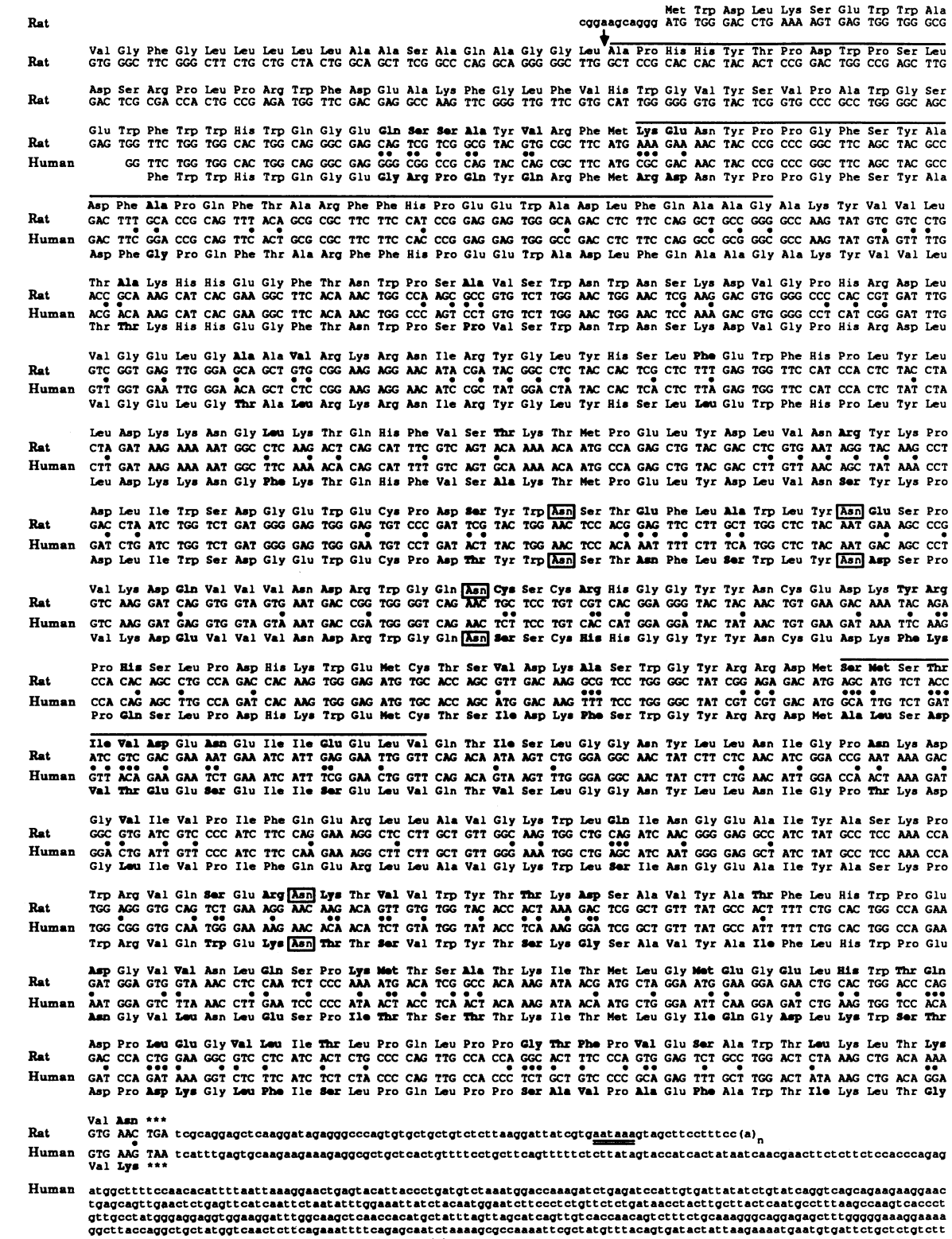

Fig. 3. Comparison of nucleotide and deduced amino acid sequences from rat and human fucosidase cDNAs

The rat fucosidase sequence is from clone FC9. The human fucosidase sequence is a composite from three partial clones [6]. Coding regions in the nucleotide sequence are given in capital letters and untranslated segments are given in lower-case letters. Nucleotide differences are indicated by black circles. Differences in the deduced amino acid sequences are shown in **bold** print. The vertical arrow represents the site of proposed cleavage by signal peptidase based on the experimentally determined *N*-terminal sequence from mature rat liver fucosidase. Continuous lines above the deduced amino acid sequence from FC9 are co-linear with amino acid sequence information obtained from CNBr-generated peptide fragments and from the *N*-terminus of mature rat liver fucosidase. Potential glycosylation sites (Asn-Xaa-Ser/Thr) are boxed. Double underlines show the potential polyadenylation signal aataaa.

Translational start site

Assignment of the first in-frame ATG noted in the rat fucosidase sequence shown in Fig. 3 as the site for translational initiation was based on the following criteria. First, although the sequence before the proposed initiation codon (CAGGG*ATG*) does not adhere exactly to the consensus sequence CC(A/G)CC*ATG* found in many eukaryotic mRNAs [19], it does satisfy the '−3 rule' by having a purine in the highly stringent −3 position relative to the initiation site. Secondly, the length of the putative signal peptide (28 residues) from the proposed start methionine residue to the *N*-terminal residue in the mature protein (Ala-29) is consistent with known signal peptide lengths [17]. In addition, this putative signal peptide is characteristic of other signal peptides in having three structurally distinct domains [20]: (i) a basic amino acid residue in the first five residues (Lys-5), (ii) a central hydrophobic region of at least nine residues (Trp-8–Ala-21), and (iii) a more polar *C*-terminal region (Ser-22–Leu-28). The open reading frame from FC9 continued in the 5′ direction beyond the proposed translational start site, and, since no studies were done to verify the functionality of the ATG at position 1, the possibility of a second upstream ATG initiating translation, albeit unlikely, cannot be ruled out. Two functional initiation sites have been reported for at least one lysosomal hydrolase [21].

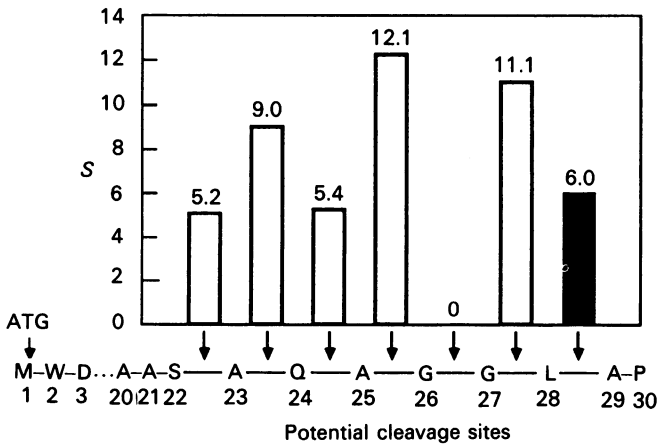

Fig. 4. Potential signal-sequence-cleavage sites in rat liver fucosidase

The *N*-terminal sequence deduced from rat fucosidase clone FC9, beginning with the first in-frame methionine residue (ATG), is given below the histogram. Vertical arrows point to potential signal-peptidase-cleavage sites. Columns in the histogram above each arrow show the probabilities (*S*) of cleavage occurring at the indicated positions according to the method of von Heijne [22]. The black column bar shows the probability of cleavage at the experimentally determined *N*-terminal residue (Ala-29) of mature fucosidase.

Limited *N*-terminal processing

N-Terminal sequence analysis of rat liver fucosidase demonstrated Ala-29 deduced from FC9 to be the *N*-terminal residue of the mature enzyme (Fig. 3). To investigate further Leu-28–Ala-29 being the site of cleavage by signal peptidase, the sequence about this site was subjected to the weighted-matrices method of von Heijne [22] for predicting signal-sequence-cleavage positions. This theoretical approach uses a window of 15 amino acid residues to scan the sequence in question for its fit to a weight-matrix composed of signal sequences with known peptidase-cleavage sites. The window position with the highest probability (*S*) is taken as the predicted cleavage site. When this method was applied to the fucosidase sequence, the window position with the highest *S* value was not that suggested by *N*-terminal sequence analysis. There were three sites that yielded higher values of *S* (Fig. 4): Ala-23–Gln-24, Ala-25–Gly-26 and Gly-27–Leu-28. Of these three alternative sites, cleavage at Ala-23–Gln-24 probably is the least likely, since it would be unusually close to the hydrophobic core of the signal peptide.

Justification of an alternative signal-peptide cleavage site from that directly predicted by the experimentally determined *N*-terminus would require that there had been limited *N*-terminal processing of fucosidase to yield Ala-29 at that end of the mature protein. Post-translational proteolytic processing is a common phenomenon among lysosomal enzymes, occurring at both the *N*- and *C*-termini, as well as within the interior of the polypeptide [23–27]. The most common processing event documented to occur at the *N*-terminus is the removal of the so-called pro-sequence from lysosomal enzymes such as hexosaminidase and cathepsin B [28,29]. If one of the three other potential signal-peptide cleavage sites is used *in vivo* and a subsequent proteolytic event generates Ala-29 at the *N*-terminus of the mature fucosidase protein, such a short one-to-five-residue clippage would be similar to the limited *N*-terminal processing recently reported for both the α- and the β-subunits of hexosaminidase [30,31]. Tollersrud & Aronson [15] also observed the *N*-terminus of a 24000-M_r subunit of the lysosomal hydrolase glycosylasparingase as being 'frayed' with one-half of this subunit population missing a single residue of serine. Asymmetric cleavage of a subunit has also been noted for the α-chain of hexosaminidase [28,30].

The lack of an obvious pro-segment larger than these one to five amino acid residues in the deduced amino acid sequence from rat liver α-L-fucosidase clone FC9 would argue against the existence of a major precursor form of the protein. Two recent studies examining the processing of α-L-fucosidase, one in human skin fibroblasts [32] and the other in human lymphocytes [33], reported no evidence for post-translational proteolysis of a precursor form of the enzyme. In both studies only a single polypeptide species having an M_r of approx. 52000 was observed. Assuming the high degree of sequence similarity between rat and human fucosidases is maintained at the *N*-terminus, the results from the above two studies are in agreement with the *N*-terminal structure measured by amino acid sequencing and deduced from the nucleotide sequence of FC9. However, the methods used to study the processing of fucosidase in cultured cells were not likely to have been sensitive enough to detect differences in M_r on the order of one to five amino acid residues.

Amino acid composition of fucosidase deduced from clone FC9

The amino acid composition of rat liver fucosidase deduced from FC9 revealed that tryptophan comprised 6% of the mature protein. This high tryptophan content

is in sharp contrast with the approximate 1% value for the average eukaryotic protein [34], although as a group lysosomal enzymes contain 2–2.5% tryptophan. The only other lysosomal hydrolase with a tryptophan content approaching that of fucosidase is α-D-galactosidase at 4% [35]. All of the 21 tryptophan residues deduced from FC9 that occur in the region of overlap between the rat and human sequences were conserved. This is not surprising considering the low mutability of this amino acid [36]. The biochemical significance of such a high tryptophan content in fucosidase remains to be determined.

The deduced amino acid sequence from FC9 showed rat liver fucosidase to have only five cysteine residues. All but one (Cys-265) are conserved in the human sequence. By using non-specific inactivators of human liver α-L-fucosidase, White *et al.* [37] showed that a non-active site cysteine residue is important for enzyme activity. Assuming that the catalytic mechanisms are similar for rat and human fucosidases, one can predict that Cys-265 present in rat fucosidase but not human fucosidase is not the residue important for enzymic activity. These four conserved cysteine residues are restricted to a relatively short stretch of 61 amino acid residues from rat fucosidase Cys-231 to rat fucosidase Cys-292, which is also the region where three of the four conserved potential glycosylation sites are located. Chemical analyses of the oligosaccharides present on purified human fucosidase indicate that on average one to three chains are present per enzyme subunit [38,39]. Thus not all of these asparagine residues are necessarily glycosylated. Barnes & Wynn [40] recently found that a Tyr-Xaa-Xaa-(Tyr/Trp/Phe) sequence is common to several lysosomal hydrolases, and they suggested that this structure may play a role in the mannose 6-phosphate targeting of these enzymes to lysosomes. Four such sequences are present in the rat fucosidase (Tyr-85, Tyr-99, Tyr-175 and Tyr-392) and they are completely conserved in the human enzyme.

Sequence similarity at the *C*-terminus of rat and human fucosidases

The 82% identity shown in Fig. 3 between rat and the incomplete human fucosidase sequences is comparable with that observed between different mammalian species for other lysosomal hydrolases [41,42]. Inspection of the amino acid changes that have occurred between the two sequences revealed they do not follow a normal distribution (χ^2 test: $0.001 < P < 0.005$). More substitutions reside in the *C*-terminal portion of the protein. Thus the identity among the 60 *C*-terminal amino acid residues is only 60%.

A recent study by Willems *et al.* [43] on the molecular defects in human fucosidosis reported the obliteration of an *Eco*RI site occurring at residues 1048–1054 of the structural gene in human α-L-fucosidase in four of 20 unrelated patients who were studied. Interestingly, this mutation occurs in the 3′-terminal region of the fucosidase gene, the area noted above as being less conserved between rat and human and therefore possibly not subjected to mutational pressures during the evolution of the two species. The sequence in the rat fucosidase gene (residues 1273–1278) that corresponds to the *Eco*RI site in the human sequence contains nucleotide substitutions at the last two positions of the 6 bp in question, both of which resulted in amino acid changes that obviously are not deleterious to the enzyme. There are only two other base-pair substitutions at these two positions that would change the amino acid sequence beyond that from either the rat or human sequences. As noted by Willems *et al.* [42], one of these substitutions would result in a termination signal (TAA), thereby possibly yielding an inactive or rapidly degraded truncated protein. If the lower degree of sequence similarity observed between rat and human at the *C*-terminus of fucosidase is an indication of the ability of this region to carry amino acid substitutions without compromising the function of the enzyme, a mutation resulting in premature translational termination (as opposed to an amino acid substitution)

Fig. 5. Comparison of the amino acid sequences deduced from rat fucosidase clone FC9 and *Dictyostelium discoideum* clone A11H2

Identical amino acid residues are indicated by black circles, equivalent amino acid residues by vertical lines. Gaps introduced to achieve optimal alignment are represented by dashes. Vertical arrows show predicted signal-peptidase-cleavage sites.

might be the best explanation for contributing to the deficiency of fucosidase in those four unrelated patients exhibiting loss of the *Eco*RI site.

Sequence similarity between rat fucosidase and a developmentally regulated gene product from *Dictyostelium discoideum*

A cDNA (A11H2) from cyclic AMP-stimulated *Dictyostelium discoideum* has recently been shown to have sequence similarity to human liver α-L-fucosidase [44]. Alignment of the *D. discoideum* sequence with that from rat fucosidase clone FC9 revealed 40% of the deduced amino acid residues to be identical (Fig. 5). The similarity between these two sequences included the *C*- and *N*-terminal portions, regions that were not compared when the *D. discoideum* sequence was compared with the incomplete human fucosidase sequence. There was, however, a lack of identity in the signal peptides from the rat and *D. discoideum* sequences. As noted by Muller-Taubenberger *et al.* [44], the *D. discoideum* cDNA sequence encodes a polypeptide having a high tryptophan content (4.3 mol%). This is also a feature of the rat and human fucosidases. Assuming that the sequence from *D. discoideum* codes for a fucosidase and that its enzymic mechanism is similar to that of rat and human fucosidases, it is noteworthy that there is only one cysteine residue that is conserved between the three species (rat enzyme Cys-292, human enzyme Cys-220 and *D. discoideum* enzyme Cys-288), and this may therefore be the cysteine residue that is important for catalysis [37].

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