Characterization of iduronate sulphatase mutants affecting N-glycosylation sites and the cysteine-84 residue

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Iduronate sulphatase (IDS) is responsible for mucopolysaccharidosis type II, a rare recessive X-linked lysosomal storage disease. The aim of this work was to evaluate the functional importance of each N-glycosylation site, and of the cysteine-84 residue. IDS mutant cDNAs, lacking one of the eight potential N-glycosylation sites, were expressed in COS cells. Although each of the potential sites was used, none of the eight glycosylation sites appeared to be essential for lysosomal targeting. Another important sulphatase co- or post-translational modification for generating catalytic activity involves the conversion of a cysteine residue surrounded by a conserved sequence C-X-P-S-R into a 2-

amino-3-oxopropionic acid residue [Schmidt, Selmer, Ingendoh and von Figura (1995) *Cell* **82**, 271–278]. This conserved cysteine, located at amino acid position 84 in IDS, was replaced either by an alanine (C84A) or by a threonine (C84T) using site-directed mutagenesis. C84A and C84T mutant cDNAs were expressed either in COS cells or in human lymphoblastoid cells deleted for the IDS gene. C84A had a drastic effect both for IDS processing and for catalytic activity. The C84T mutation produced a small amount of mature forms but also abolished enzyme activity, confirming that the cysteine residue at position 84 is required for IDS activity.

INTRODUCTION

Iduronate sulphatase (IDS; EC 3.1.6.13; L-iduronate-2-sulphate 2-sulphohydrolase) is one of the lysosomal enzymes involved in the degradation of heparan sulphate and dermatan sulphate. IDS deficiency is responsible for mucopolysaccharidosis type II, a rare X-linked lysosomal storage disease [1]. A 2.3 kb cDNA clone containing the complete coding region of IDS was isolated, and the reading frame contains 550 amino acids [2]. Normal IDS processing has been studied after IDS cDNA overexpression and endocytosis in fibroblasts [3], and the processing steps were found to be identical in COS cells and in lymphoblastoid cell lines (L β) [4]. In our study, the functional importance of each N-glycosylation site and of the conserved cysteine-84 residue was evaluated.

Sequence analysis of IDS cDNA revealed the presence of eight potential N-linked glycosylation sites (Asn-X-Ser/Thr) at positions 31, 115, 144, 246, 280, 325, 513 and 537 (Figure 1). Eight IDS mutant cDNAs lacking selected N-glycosylation sites were prepared by site-directed mutagenesis and expressed in COS-7 cells. The processing of these mutants was studied and their catalytic activity measured to evaluate the functional importance of each of these glycosylation sites.

Another potentially important co- or post-translational modification for all sulphatases is the conversion of a conserved cysteine residue into a 2-amino-3-oxopropionic acid residue. The deficiency of this conversion has been demonstrated for arylsulphatase A in MSD (multiple sulphatase deficiency) fibroblasts [5]. Using site-directed mutagenesis, the conserved cysteine-84 of IDS was replaced by either an alanine (C84A) or a threonine (C84T). These two mutants IDS cDNAs were expressed either in COS-7 cells or in lymphoblastoid cells and analysed for processing and catalytic activity. IDS processing and activity were also studied in the MSD L β stably transfected with the normal IDS cDNA.

MATERIALS AND METHODS

Materials

Tritiated disaccharide substrate O-(α -L-idopyranosyluronic acid 2-sulphate)-(1 $\rightarrow$ 4)-2,5-anhydro-D-[³H]mannitol 6-sulphate was purchased from Professor Krasnopolskaya (University of Mos-

Figure 1 Construction of IDS cDNA lacking individual potential glycosylation sites

Positions of the eight glycosylation sites (N31, N115, N144, N246, N280, N325, N513, N537) are indicated. Eight mutant cDNAs (N31Q, N115Q, N144Q, N246Q, N280Q, N325Q, N513Q, N537Q) lacking one potential glycosylation site were constructed.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EndoH, endoglycosidase H; IDS, iduronate sulphatase; L β , lymphoblastoid cell line; MSD, multiple sulphatase deficiency; PNGaseF, N-glycosidase F.

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Table 1 Oligonucleotides used for mutagenesis

The altered codons are indicated in bold, italic characters.

Oligonucleotides	Sequences
N31Q	5'-GAAACGCGAGGCC CAG TCGACCACAGAT-3'
N115Q	5'-GTGCACGCTGGAC CAG TTTCTCCACCATC-3'
N144Q	5'-GGGATATCTTCT CAG CATACCGATGAT-3'
N246Q	5'-TATCCCTTGGAG CAG ATCACCTCGCC-3'
N280Q	5'-GTCCAAGCCTTA CAG ATCAGTGTGCCG-3'
N325Q	5'-CTTCAGCTGGCC CAG AGCACCATCATT-3'
N513Q	5'-GAATTTCTAGCT CAG TTTCTGACATC-3'
N537Q	5'-CACAATATGTAT CAAG ATTCCCAAGGT-3'
C84A	5'-CGCAGCAAGCAGT GCCG CCCCGAGCCGCG-3'
C84T	5'-CGCAGCAAGCAGT ACC GCCCCGAGCCGCG-3'
MUT- <i>Xho</i> I	5'-GCTCGACGGTACTCGCGGAAGTGAATAAC-3'

cow, Russia). The full-length IDS cDNA clone (pB2Sc17) was kindly provided by Professor J. J. Hopwood (Adelaide Children's Hospital, North Adelaide, South Australia 5006, Australia). U.S.E. mutagenesis kit and Protein A-Sepharose CL-4B were from Pharmacia. pTK vector was from Clontech. Dulbecco's modified Eagle's medium (DMEM)/25 mM Hepes was from Gibco. PMSF and tunicamycin were from Sigma. Reagents for SDS/PAGE and the Gene Pulser electroporation unit were from Bio-Rad. Sequenase Quick-Denature Plasmid Sequencing Kit, Amplify, and Rainbow ¹⁴C-labelled methylated protein molecular-mass markers were from Amersham. Tran ³⁵S label and DMEM deficient in methionine and cysteine were from ICN. Endoglycosidase H (EndoH) and N-glycosidase F (PNGaseF) were from BioLabs.

Cell lines

Deleted *L*βs were from a mucopolysaccharidosis-type-II patient presenting a complete deletion of the IDS gene [6].

MSD *L*βs were from a patient affected by MSD. The biochemical diagnosis was assessed by demonstrating the deficiency of several sulphatases in skin fibroblasts: arylsulphatases A and B, iduronate-2-sulphatase, heparan sulphamidase, N-acetylglucosamine-6-sulphate sulphatase and steroid sulphatase.

*L*β and COS cells were cultivated in RPMI 1640 and in DMEM/25 mM Hepes respectively supplemented with 12% (v/v) fetal-calf serum and antibiotics at 37 °C in a humidified air/CO₂ (19:1) incubator.

Transfection procedure

*L*β were electroporated to enhance transfection with a eukaryotic vector containing the full-length human IDS cDNA under the control of the Rous Sarcoma Virus (RSV) promoter (pREP-IDS) [7]. The experimental conditions were: 5 × 10⁶ cells in 500 μl of RPMI 1640/25 mM Hepes, 50 μg of plasmid DNA (pREP-IDS) and a voltage pulse of 250 V, 960 μF. At 48 h post-electroporation, the cells were selected with 50 units/ml hygromycin B. Resistant cell lines were maintained in complete RPMI 1640 medium containing 50 units/ml hygromycin B.

COS cells were electroporated to enhance transfection with a eukaryotic vector containing the normal (pTK-IDS) or mutated IDS cDNA under the control of the thymidine kinase promoter. The experimental conditions were: 20 × 10⁶ cells in 500 μl of DMEM/25 mM Hepes, 100 μg of plasmid DNA and a voltage pulse of 220 V, 960 μF.

Figure 2 Digestion of IDS polypeptides by EndoH or PNGaseF in transfected COS cells

COS cells transfected with wild-type cDNA were labelled for 3 h (pulse) and harvested directly or after the indicated chase period. IDS polypeptides were immunoprecipitated from cell homogenates, incubated with either EndoH or PNGaseF, and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left and the right.

Constructs

IDS mutants were generated by site-directed mutagenesis using the U.S.E. mutagenesis kit (Pharmacia). Each mutant was constructed using one single-stranded vector (pTK-IDS or pREP-IDS) and two oligonucleotides: one mutagenic oligonucleotide containing the desired mutation and the MUT-*Xho*I oligonucleotide to abolish the unique *Xho*I restriction site in the vector for selection of mutated plasmids. The sequences of these oligonucleotides are indicated in Table 1. For each of the eight glycosylation sites, the asparagine codon (Asn) was converted into a glutamine codon (Gln). The mutated plasmids were sequenced by the dideoxy chain-termination method [8].

Pulse-chase labelling, immunoprecipitation, glycosidase digestions and IDS activity measurements

Experimental conditions have been previously described [3].

RESULTS

Functional characterization of IDS N-glycosylation sites

Glycosylation studies (Figure 2)

COS cells transfected with the wild-type cDNA produced a 76 kDa precursor that was rapidly converted into a smeary phosphorylated 90 kDa form (3 h pulse). The 90 kDa precursor is converted into a major intermediate 62 kDa form giving the 55 kDa (major form) and 45–44 kDa (doublet) mature polypeptides (24 h chase). These ³⁵S-labelled IDS polypeptides were digested by either EndoH or PNGaseF. After EndoH or PNGaseF digestion, the 76 kDa band was converted into a unique 60 kDa band; this precursor contains only hybrid and/or high-mannose-type oligosaccharide chains. After digestion by EndoH, the smeary 90 kDa band was converted into a band with a slightly lower molecular mass, while, after PNGaseF digestion, a unique 60 kDa band was observed: the 90 kDa precursor contains some hybrid and/or high-mannose glycans and mostly complex glycans. The 62 kDa intermediate and 55 kDa mature form were digested to a 48 kDa form by EndoH and to a

Figure 3 IDS pulse–chase labelling and IDS activity in COS cells transfected with N-glycosylation mutant cDNAs

(A) COS cells transfected with wild-type (WT) and mutant cDNA constructs were labelled for 3 h (pulse) and harvested directly, or after a 24 h chase. IDS polypeptides were immunoprecipitated from cell homogenates and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left and the right. (B) COS cells were harvested 48 h after transfection. Cell extracts and media were assayed for IDS activity. One unit is defined as the amount of enzyme that releases 1 pmol of tritiated disaccharide substrate per hour. IDS activity values are the averages of three different transfections in which the basal activities of COS cells were subtracted.

46 kDa form by PNGaseF. The 45–44 kDa form was converted into a 41–40 kDa form after EndoH digestion, and into a 39–38 kDa form after PNGaseF digestion. These three IDS polypeptides contain both hybrid, high-mannose, and at least one complex-type, oligosaccharide chains.

Functional characterization of each N-glycosylation site (Figure 3)

³⁵S-labelled wild-type and mutant IDS polypeptides were immunoprecipitated from cell homogenates after a 3 h pulse period or after a subsequent chase of 24 h, and analysed by SDS/PAGE (Figure 3A). After a 3 h ³⁵S-labelling period, all mutant precursor forms showed a slightly lower molecular mass (74 kDa) than the wild-type precursor (76 kDa): each potential N-glycosylation site was glycosylated.

After a 24 h chase period, the 74 kDa precursor form of the N31Q, N513Q and N537Q IDS mutants were processed to the normal 55 kDa and 45–44 kDa mature forms (Figure 3A). N115Q, N144Q, N246Q, N280Q and N325Q IDS mutants were processed to lower-molecular-mass mature forms of 53 kDa and 44–43 kDa, but the ratio of the 43 kDa mature form to the 53 kDa mature forms was higher for the N325Q IDS cDNA mutant.

IDS activity was measured in cell extracts and in the media of transfected COS cells. Cells transfected either by the wild-type cDNA or by an N-glycosylation mutant showed a striking increase in IDS activity compared with the basal IDS activity of COS cells (150–200 pmol·h⁻¹·mg⁻¹) (Figure 3B). The lowest activities were observed for mutants N144Q and N246Q. In the media, the percentage of secreted IDS activity was similar in the

Table 2 IDS activities in stably transfected L β

Cells	Constructs	IDS activity (pmol · h ⁻¹ · mg ⁻¹)
Deleted L β	C84A	0
Deleted L β	C84T	0
Deleted L β	Wild-type	5464
Deleted L β	Vector only	0
MSD L β	Wild-type	0
MSD L β	Vector only	0
Normal L β	Vector only	29

Table 3 IDS activities in transiently transfected COS cells

Cells	Constructs	IDS activity (pmol · h ⁻¹ · mg ⁻¹)
COS cells	C84A	169
COS cells	C84T	144
COS cells	Wild-type	3985
COS cells	Vector only	132

Figure 4 ³⁵S pulse–chase labelling in L β stably transfected with C84A or C84T mutant cDNAs

L β stably transfected with wild-type, C84A and C84T constructs were labelled for 3 h (pulse) and harvested directly or after a 24 h chase. IDS polypeptides were immunoprecipitated from cell homogenates and separated by SDS/PAGE. Exposure time was 6 days, except for cells transfected with the wild-type cDNA (2 days). Positions of molecular-mass markers are shown on the left.

wild-type and in the mutants, except the N280Q mutant, for which a 3-fold increase was observed.

Functional analysis of C84A and C84T IDS mutant cDNAs

Transfection of C84A and C84T IDS cDNA mutants in L β and in COS cells

After transfection of COS cells or L β with C84A or C84T IDS cDNAs, no significant increase in IDS activity could be observed, but after transfection with normal IDS cDNA, activity reached 30 times normal in COS cells and 200 times normal in L β (Tables 2 and 3).

The 76 kDa precursor produced in C84A L β was poorly converted into the 90 kDa form (pulse 3 h), leading to an

Figure 5 ^{35}S pulse-chase labelling in COS cells transiently transfected with C84A or C84T mutant cDNAs

COS cells transiently transfected with wild-type, C84A and C84T constructs were labelled for 3 h (pulse) and harvested directly or after a 24 h chase. IDS polypeptides were immunoprecipitated from the cell homogenates and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left.

Figure 6 ^{35}S pulse-chase labelling of MSD L β and deleted L β stably transfected with the wild-type IDS cDNA

MSD and deleted L β stably transfected with wild-type cDNA were labelled for 3 h (pulse) and harvested directly or after the indicated chase period (h). IDS polypeptides were immunoprecipitated from cell homogenates and separated by SDS/PAGE. Positions of molecular-mass markers are shown on the left.

important decrease in the mature polypeptides produced (chase 24 h) (Figure 4). In L β stably transfected with the C84T cDNA, the amount of the 76 kDa precursor processed into the 90 kDa form was also decreased, but not as much as in C84A L β . Consequently, L β expressing C84T cDNA produced more mature forms than L β expressing C84A cDNA.

Similar results were observed in COS cells transiently transfected with either the C84A cDNA or the C84T cDNA (Figure 5).

Transfection of MSD L β with the normal IDS cDNA

In non-transfected MSD L β , no IDS activity could be measured even after a long-term culture. After transfection of MSD L β with the wild-type IDS cDNA, activity remained undetectable (Table 2). Pulse-chase experiments showed, after a 3 h ^{35}S -labelling period, the presence in normal amount of the 76 kDa precursor form poorly converted into 90 kDa forms (Figure 6). After a 24 or 48 h chase, the 90 kDa precursors were converted into 55 and 45 kDa mature forms.

DISCUSSION

IDS undergoes several post-translational modifications during its transport to lysosomes. The initial glycosylated 76 kDa precursor form is converted into a phosphorylated 90 kDa form (Scheme 1). This form is then processed by other glycosylation modifications and proteolytic cleavages through various intermediates to a major 55 kDa mature form. Further partial proteolytic cleavage gives the 45 kDa mature form.

Functional characterization of IDS N-glycosylation sites

Asparagine residues in the sequence Asn-X-Ser/Thr (X = any amino acid but Pro) are the potential sites for attachment of N-linked oligosaccharide chains transferred *en bloc* from the dolichol pyrophosphate. Site-directed mutagenesis of each potential glycosylation site was used to establish the number of sites used and their functional role. A molecular-mass decrease of approx. 2 kDa (corresponding to the removal of one carbohydrate chain) was observed for all mutant precursor forms, showing that each potential N-glycosylation site was used. Pulse-chase experiments showed that all mutant precursors were correctly processed to the mature forms: none of the glycosylation sites was essential for lysosomal targeting of IDS. Similar results were reported for some lysosomal enzymes [9–11], but not for all of them: α -glucosidase was not processed if the second glycosylation site at Asn-233 was abolished [12].

IDS activity of cells transfected with the various mutants was close to that obtained with the wild-type cDNA. IDS activity secreted in the medium by the N280Q mutant was 3-fold higher than the wild-type: this suggests that this glycosylation site may be the most important for lysosomal targeting. A preferential phosphorylation of this glycosylated site could account for that result, as it has been suggested for β -glucuronidase [9]. The four N-glycosylation sites of β -glucuronidase were glycosylated, but the absence of the preferentially phosphorylated sites 2 and 3 resulted in very reduced amounts of the lysosomal form and increased secretion [9].

Study of IDS cDNA mutants lacking two or more glycosylation sites would probably result in a cumulative effect on processing and enzyme activity, as shown for β -glucuronidase [9]. Unglycosylated IDS precursors synthesized in the presence of tunicamycin were not processed and inactive, showing that N-glycosylation is required for processing and enzyme activity [4]. Unglycosylated precursors of lysosomal enzymes are probably misfolded and degraded in the endoplasmic reticulum [13].

Comparison of the mobility shifts of the mutant precursor and mature forms confirmed the localization of some of the proteolytic sites. Three IDS cDNA mutants (N31Q, N513Q or N537Q) synthesized the normal 55 kDa mature form: processing from 76 to 55 kDa involves an N-terminal proteolytic step

Scheme 1 IDS processing in COS cells

Molecular masses of deglycosylated polypeptides are indicated in parentheses (kDa).

downstream of Asn-31, and a C-terminal proteolytic step upstream of Asn-513. As previously described [2], the N-terminal proteolytic pre-lysosomal cleavage removes a propeptide of eight amino acids (amino acids 26–33) and occurs after the removal of the 25-amino-acid N-terminal signal sequence. The C-terminal proteolytic cleavage (Scheme 1) (C-proteolysis 1) produces the 62 kDa intermediate form and 18 kDa polypeptide [3]. The 18 kDa polypeptide has been previously sequenced [2] and the N-terminal identified at amino acid residue 456.

The further proteolytic step was the conversion of the 55 kDa mature form into the 45–44 kDa mature polypeptides. As indicated by the molecular mass of these deglycosylated forms, this second proteolytic step led to the release of a 7–8 kDa polypeptide (Scheme 1). This step is probably a C-terminal proteolytic cleavage (C-proteolysis 2), because the removal of 7–8 kDa from the N-terminal sequence of the 55 kDa form would eliminate the highly conserved sequence C⁸⁴-X-P-S-R⁸⁸.

Functional analysis of two site-directed mutations affecting Cys-84 of the IDS gene

Another important sulphatase co- or post-translational modification involves the conversion of a cysteine residue surrounded by a conserved sequence (C-X-P-S-R) into a 2-amino-3-oxopropionic acid residue [5]. Schmidt et al. [5] have proposed that this conversion of the cysteine is required for the catalytic activity of sulphatases. Its deficiency would be the cause of MSD, as structural analysis of arylsulphatase A and arylsulphatase B from MSD fibroblasts demonstrated that cysteine-to-serine semi-aldehyde conversion was deficient. In this rare autosomal recessive disorder, all sulphatase activities are severely decreased, despite a normal processing [14].

This conserved cysteine, located at amino acid position 84 in IDS, was replaced either by an alanine (C84A) or by a threonine (C84T). Cysteine and threonine are both polar amino acids, whereas alanine is hydrophobic. C84A and C84T mutant cDNAs were expressed in COS cells and in deleted L β . The C84A substitution had a drastic effect both for IDS processing and for catalytic activity. The C84T mutation produced a small amount of mature forms but also abolished the enzyme activity, confirming that the cysteine residue is required for IDS activity. MSD L β stably transfected with the wild-type IDS cDNA gave results similar to the C84T mutant. Previous expression studies of arylsulphatase A, arylsulphatase B and steroid sulphatase in MSD fibroblasts showed that polypeptides were normally processed but inactive, suggesting that a co- or post-translational modification of sulphatases is required for their catalytic activity [14]. In transfected MSD L β , IDS precursors were inactive, in contrast with IDS precursors secreted in the medium of over-expressing deleted L β that were catalytically active *in vitro* [4]. This result is in accordance with the hypothesis of Schmidt et al.

[5], who suggested that this modification occurs as an early post-translational or co-translational event, most probably in the endoplasmic reticulum.

Previous studies have reported that the residual activities of arylsulphatases A, B and C vary in MSD fibroblasts and can be affected by culture conditions such as pH, medium composition and culture time [15]. A varying IDS activity ranging from 3 % to 25 % of mean normal value was measured in our patient's fibroblasts at different times (results not shown). In contrast, no IDS activity could be observed in untransfected and transfected L β from the same MSD patient (even after a 10-month culture), whereas arylsulphatases A and B showed a variable residual activity. These results suggest that the saturable mechanism of conversion varies from one cell type to another and has a greater affinity for arylsulphatases A and B than for IDS in L β .

The functional characterization of IDS mutants affecting the N-glycosylation sites and the cysteine-84 residue substantiated the knowledge of IDS maturation and confirmed, for IDS, the importance of the modification of the conserved cysteine for sulphatase activity.

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