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# COS cell expression studies of P86L, P86R, P480L and P480Q Hunter's disease-causing mutations

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**Abstract**

Three missense mutations identified in the *IDS* gene of our Hunter's disease patients (P86L, P480L and P480Q) and the previously described P86R mutation were expressed in COS cells to evaluate their functional consequence on iduronate-2-sulfatase (IDS) activity and processing. The 86-proline residue belongs to the highly conserved pentapeptide C-X-P-S-R in which cysteine modification to a formylglycine is required for sulfatase activity. The substitution of the 86-proline residue led to a severe mutation as no mature form was targeted to the lysosome in agreement with the severe phenotype observed in patients carrying P86L and P86R mutations. Expression studies with P480L and P480Q mutant cDNAs showed the presence of a small amount of 55 kDa mature form in the lysosomes of transfected COS cells. IDS activity of the P480L and P480Q mutants in cell extracts represents 16.6% and 5.4% of the wild-type, respectively. © 1998 Elsevier Science B.V.

*Keywords:* Iduronate-2-sulfatase (IDS); Mucopolysaccharidosis; Hunter's disease; Expression study; Lysosome

## 1. Introduction

Iduronate-2-sulfatase or IDS (EC 3.1.6.13; L-iduronate-2-sulfate 2-sulfohydrolase) is one of the lysosomal enzymes involved in the degradation of heparan sulfate and dermatan sulfate. IDS deficiency is responsible for mucopolysaccharidosis type II (MPS II), a rare X-linked lysosomal storage disease [1]. A broad spectrum of clinical phenotypes can be observed, ranging from the mild form with late onset and fairly normal intelligence to the severe form with mental retardation and early death.

Human IDS has been purified [2] and a 2.3-kb cDNA clone containing the complete coding region was isolated and the reading frame contains 550 amino acids [3]. The gene spans approximately 24 kb and contains 9 exons [4,5]. IDS processing steps have been described in different cell lines: the 76-kDa precursor is processed through various intermediates forms to the 55-kDa and 45-kDa mature forms [6,7].

Three missense mutations were identified in the *IDS* gene of our patients: one previously described (P86L) [8] and two unreported (P480L and P480Q). These three mutations and the previously described P86R mutation [9,10] were expressed as the substitutions of these two different prolines led to different clinical phenotypes: a severe phenotype for the proline 86, and a mild phenotype for the proline 480. These four mutations were expressed in COS cells to evaluate their functional consequence on IDS activity and processing.

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## 2. Materials and methods

### 2.1. Materials

Tritiated disaccharide substrate 0-(α-L-idopyranosyluronic acid 2-sulfate)-(1 → 4)-2,5-anhydro-D-$^{3}$H-mannitol-6-sulfate was purchased from Prof. Krasnopolskaya (University of Moscow, Russia). The full-length IDS cDNA clone (pB2Sc17) was kindly provided by Prof. J.J. Hopwood (Adelaide Children's Hospital, North Adelaide, 5006 South Australia, 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. Phenylmethyl-sulfonyl fluoride (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, Thermosequenase cycle sequencing kit, Amplify, and Rainbow $^{14}$C methylated protein molecular-mass markers were from Amersham. Tran $^{35}$S-label and DMEM deficient in methionine and cysteine were from ICN.

### 2.2. Patients: clinical data and identification of mutations

#### 2.2.1. Clinical data

Patients' clinical data are presented in Table 1. MPS II diagnosis was assessed by demonstrating a high excretion of heparan sulfate and dermatan sulfate in urine and an undetectable IDS activity in leukocytes and fibroblasts.

#### 2.2.2. Identification of the mutations

Total RNA was extracted from fibroblasts by guanidium isothiocyanate/cesium chloride gradient procedure [11] and converted to cDNA using the first-strand cDNA synthesis kit (Pharmacia). IDS cDNAs were PCR-amplified in two fragments (A and B) using specific oligonucleotide primers [3]:

A (692 bp): primer 1 (5′ $^{32}$ACGAGGAGGTCTCTGTGGCT$^{51}$)/primer 1′ (3′ $^{580}$CGTGGAACGGACTGTTTGTCT$^{600}$ 5′)B (123o bp):primer 2 (5′ $^{624}$CACTAAGACATGTCGAGGGC$^{644}$ 3′)/primer 2 (3′ $^{1833}$CCTCAATCTCGACCAGCAAAAC$^{1854}$ 5′)

After amplification, PCR products were purified on a 8% or 5% acrylamide gel and directly sequenced with the Thermosequenase cycle sequencing kit using [γ $^{33}$P]-ATP.

Genomic DNA was extracted from fibroblasts or peripheral blood leucocytes to verify the presence of the mutation [12].

### 2.3. Expression studies

#### 2.3.1. Constructs

IDS cDNA mutants were generated by site-directed mutagenesis using the U.S.E. mutagenesis kit. Each mutant was constructed using one single-stranded vector (pTK–IDS containing the normal IDS cDNA) 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 oligo-

Table 1
Patient's data

| Case | Patient 1 | Patient 2 | Patient 3 |
|---|---|---|---|
| Phenotype | Severe | Mild | Mild |
| Age (at last examination) | 13 yrs | 18 yrs | 45 yrs |
| Mental retardation | + + + | − | − |
| Coarse facies | + + + | −/+ | −/+ |
| Joint stiffness | + + | −/+ | + |
| Dysostosis | + + | − | + + |
| *Genotype* | | | |
| Mutation | P86L (381 C → T) | P480L (1563 C → T) | P480Q (1563 C → A) |
| Exon | III | IX | IX |

Table 2
Oligonucleotides used for mutagenesis

| Oligonucleotides | Sequences |
|---|---|
| P86L | 5′ GCAGTGTGCGCCCTGAGCCGCGTTTC 3′ |
| P86R | 5′ GCAGTGTGCGCCCGGAGCCGCGTTTC 3′ |
| P480L | 5′ GGAATTCTGACAAGCTGAGTTTAAAAGA 3′ |
| P480Q | 5′ GGAATTCTGACAAGCAGAGTTTAAAAGA 3′ |
| Mut-*Xho*I | 5′ GCTCGACGGTACTCGCGGAACTGAAAAACC 3′ |

nucleotides are indicated in Table 2. The sequence of mutated plasmids was verified by using the kit.

*2.3.2. Transfection procedure*

COS cells were cultivated in DMEM–25 mM HEPES, supplemented with 1 mM pyruvate sodium, 12% (v/v) fetal calf serum and antibiotics. COS cells were electroporated with an eukaryotic vector containing the normal (pTK–IDS) or mutated IDS cDNA under the control of the Thymidine Kinase promoter. The experimental conditions were: $20.10^6$ cells in 500 μl of DMEM–25 mM HEPES, 100 μg of plasmid DNA and a voltage pulse of 220 V, 960 μF.

*2.4. Pulse-chase labelling, immunoprecipitation and IDS activity measurement*

Experimental conditions have been previously described [6].

## 3. Results

*3.1. Identification of the mutations*

The severely affected patient 1 has a C → T transition at nucleotide 381 of the cDNA that produces Pro → Leu substitution in codon 86. This mutation partially activates a cryptic splice acceptor site (28 bp downstream the mutation) leading to two different transcripts: one transcript containing the P86L mutation, and another with a deletion of 44 bp starting at nucleotide 365 of the cDNA (data not shown). This deletion changes two amino acids and creates a stop codon in codon 83. This truncated polypeptide is unable to give any mature form as reported for other nonsense mutations in the *IDS* gene [6,13]. The mildly affected patients 2 and 3 have a C → T transition and a C → A transversion at nucleotide 1563 of the cDNA leading to Pro → Leu and Pro → Gln substitutions in codon 480, respectively. These mutations identified both by sequencing of the cDNA and the genomic DNA were also confirmed by allele-specific oligonucleotide hybridization.

*3.2. Expression of IDS mutant cDNAS*

P86L, P86R, P480L and P480Q mutants were transiently expressed in COS cells. $^{35}$S-labeled wild-type and mutant IDS polypeptides were immunoprecipitated from cell homogenates after a 3-h pulse period or after a subsequent 24-h chase, and analysed by SDS-PAGE (Fig. 1). After a 3-h pulse, COS cells

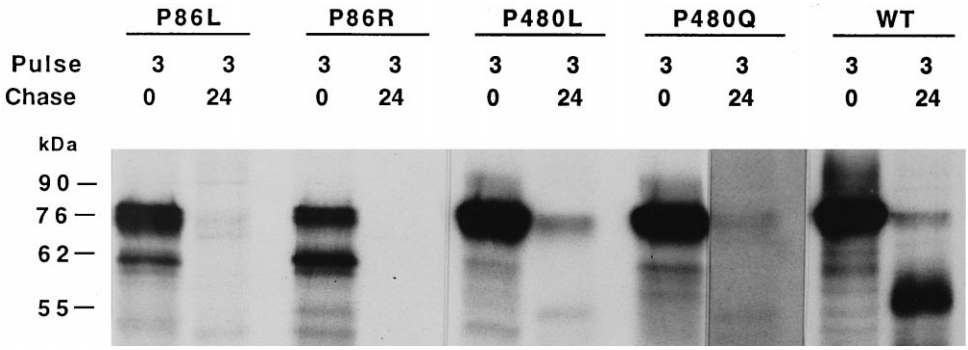


Fig. 1. $^{35}$S Pulse-chase labeling of COS cells transiently transfected with wild-type and IDS mutant cDNAs. Exposure time was 4 days except for P480Q cell extracts after a 24-h chase (20 days).

Table 3
IDS activity in COS cells transfected with wild-type and mutant cDNAs

| Constructs | IDS activity (pmol $h^{-1}$ $mg^{-1}$) |
|---|---|
| P86L | $151 \pm 24$ |
| P86R | $143 \pm 10$ |
| P480L | $758 \pm 129$ |
| P480Q | $338 \pm 50$ |
| WT | $3891 \pm 385$ |
| Mock | $136 \pm 10$ |

IDS activity values are the average of four different transfections

transfected with either the P86L or the P86R mutant cDNA produced the 76-kDa precursor rapidly converted to a 62-kDa form (Fig. 1). No 90-kDa precursor could be visualized. After a 24-h chase, no 55-kDa mature form was produced while the 76- and 62-kDa polypeptides were degraded. No increase of IDS activity in these transfected cells was observed (Table 3).

The 76- and 90-kDa precursor forms were visualized after a 3-h labeling period in COS cells transfected with either the P480L or the P480Q mutant cDNA (Fig. 1). A small amount was processed to the 55-kDa mature form after a 24-h chase. More 55-kDa mature form was produced by P480L than by P480Q mutant. In these transfected cells, IDS activity produced by the P480L and P480Q mutants represents 16.6% and 5.4% of the wild-type, respectively (Table 3).

## 4. Discussion

The functional importance of the prolines at position 86 and 480 was investigated as the substitutions affecting these prolines led to a severe and a mild phenotype, respectively.

In cells transfected with the P86L and P86R IDS mutant cDNAs, no lysosomal mature forms and no increase of IDS activity could be detected. These results are in agreement with the severe phenotype observed in patient 1 and others with the P86L or P86R mutations [8–10]. The P86L and P86R mutations affect the proline residue of the C-X-P-S-R sequence highly conserved in all sulfatases [14]. In this pentapeptide, the early conversion in the endoplasmic reticulum of the cysteine to a formylglycine is required for sulfatase activity and its deficiency causes multiple sulfatase deficiency (MSD) [15,16]. Expression studies performed on MSD cells showed that the absence of the modification does not prevent maturation of arylsulfatase A and IDS precursor forms [17,18]. Similar results were obtained when this conserved cysteine was mutated either to a serine residue in *N*-acetylgalactosamine 4-sulfatase [19] or to a threonine residue in IDS [18]. In contrast to this cysteine residue, the conserved proline residue, close or even part of the IDS active site appears as essential for the proper folding and processing of IDS.

The 480 proline residue is localized in a poorly conserved region of the *IDS* gene. Expression studies showed that the P480L and P480Q precursor forms were very poorly processed to the lysosomal 55-kDa mature form compared to COS cells transfected with the wild-type IDS cDNA. IDS activity of the P480L and P480Q mutants represents 16.6% and 5.4% of the wild-type respectively, when measured in cell extracts. As IDS precursors are catalytically active in vitro [7], subcellular fractionation was performed to evaluate the percentage of IDS activity in the lysosomes of transfected COS cells. No significant increase of IDS activity could be measured in the lysosomal fraction of these transfected cells when compared to the basal activity of COS cells lysosomes because of the small amount of mature forms produced by these mutants. IDS activity measurement in patient cells cannot discriminate between a complete deficiency and a very low residual IDS activity, but a low enzyme activity is enough to allow a normal phenotype in most lysosomal diseases [20]. The small amount of the 55-kDa lysosomal mature form observed in Fig. 1 probably retains enough activity to confer a mild phenotype to patients 2 and 3.

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