Heparan *N*-sulfatase: cysteine 70 plays a role in the enzyme catalysis and processing

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Abstract Sulfatases are members of a highly conserved family of enzymes that catalyze the hydrolysis of sulfate ester bonds from a variety of substrates. The functional correlation reflects a high degree of amino acid sequence similarity along the entire length, in particular in the active site where the C(X)PSR consensus sequence is present. Cysteine undergoes an important co- or post-translation modification essential for the accomplishment of catalytic activity: conversion in formylglycine. In this work, the cysteine of heparan *N*-sulfatase (NS) was replaced either by a serine (C70S) or by a methionine (C70M) using site-directed mutagenesis. C70S and C70M mutant cDNAs were expressed and analyzed in COS cells; both mutations caused a loss of NS activity; however, while C70S showed a normal precursor form undergoing processing to a reduced mature form within the lysosomes, C70M was poorly synthesized and formed a complex with the molecular chaperone immunoglobulin binding protein.

Key words: Heparan *N*-sulfatase; Mucopolysaccharidosis IIIA; Protein processing; Immunoglobulin binding protein

1. Introduction

Heparan *N*-sulfatase (NS, EC 3.10.1.1) is a lysosomal enzyme involved in the degradation of heparan sulfate; the deficiency of this enzyme [1,2] results in mucopolysaccharidosis type IIIA (MPS-IIIA), an autosomal recessive disorder characterized by clinical severity [3]. The full-length cDNA and genomic sequences for human NS have been isolated and characterized [4,5]; the NS gene is located on chromosome 17q25.3. Molecular analysis performed in MPS-IIIA patients allowed the identification of a large variety of alterations, with some prevalent mutations and several other defects providing evidence for clinical heterogeneity [6–9]. In humans, NS has been characterized in different tissues as a 56 kDa polypeptide [10]; the recombinant NS protein is synthesized in the rough endoplasmic reticulum (RER) as a 62 kDa glycosylated precursor protein that is targeted through Golgi and processed in the vacuolar network until it reaches the lysosomes, where it is detected as a 56 kDa mature glycoprotein [11,12]. The sequence contains five putative *N*-glycosylation sites, which we have recently demonstrated to be all functional, with Asn41 and Asn151 having a role in the protein folding and/or stability [13].

NS is a member of an evolutionary conserved gene family that hydrolyze sulfate ester bonds from a variety of substrates including glycosaminoglycans, glycolipids and steroids. Eleven mammalian sulfatases have been identified: DNA alterations on eight of these cause distinct human disorders. Mutations affecting sulfatases responsible for mucopolysaccharidoses have recently been reviewed [14]. The homology of the sulfatase sequence is striking, especially in the N-terminal region, where the active site characterized by the conserved amino acid sequence C(X)PSR is located [15]. Besides sequence homology, sulfatases also share a unique post-translational modification that converts the conserved cysteine into formylglycine in the endoplasmic reticulum [16]. The lack of this modification leaves newly synthesized sulfatases inactive and is the cause of multiple sulfatase deficiency (MSD), a rare but fatal lysosomal storage disorder where all sulfatase activities are severely decreased despite normal processing [17]. Crystallographic analysis has demonstrated the structural similarity of the sulfatases and confirmed that the conserved cysteine residue is part of the catalytic site [18].

In this study, we produced by site-directed mutagenesis in vitro two NS mutant cDNAs in which the highly conserved cysteine was replaced either by a serine or by a methionine; each recombinant mutant cDNA was transfected in COS cells and analyzed for the NS enzymatic activity and biosynthesis. We show that position 70 is critical for enzyme activity and processing: the C70S mutant protein is secreted in the medium, processed to the mature form in the lysosomes but is partially degraded; the C70M mutant protein is not secreted and forms a complex with BiP, an immunoglobulin heavy chain binding protein.

2. Materials and methods

2.1. Site-directed mutagenesis

NS cDNA mutants were generated by site-directed mutagenesis using the Transformer II kit (Clontech) according to the manufacturer's instructions.

To introduce the mutations C70S and C70M, the following oligonucleotides were used: 5′ (CTCGGTCAGCAGCAGCTCTCCCAGCCGCGC, C70S); 5′ (CTCGGTCAGCAGCATGTCTCCCAGCCGCGC, C70M).

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Abbreviations: MPS-IIIA, mucopolysaccharidosis type IIIA; NS, heparan *N*-sulfatase; wt, wild-type; BiP, immunoglobulin binding protein; RER, rough endoplasmic reticulum

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2.2. Transfection procedure, NS and β-galactosidase activity measurements, Western blot analysis, metabolic labeling, reverse transcriptase-PCR (RT-PCR)

The conditions for these procedures have been previously reported [9].

2.3. Association of NS proteins to BiP

Forty eight hours after transfection, COS cells in 6 cm plates containing wild-type (wt) or mutant cDNA constructs were immunoprecipitated with 2 μl anti-NS antibody. The immunoprecipitated proteins were denatured, separated by 10% SDS–PAGE and transferred to nitrocellulose filters (Bio-Rad). The membranes were treated with a monoclonal antibody recognizing BiP (Grp94 and Grp78) used at a 1:500 dilution in TBS with 1% dry milk; the antibody immunoprecipitates also an aspecific protein of 40 kDa (SPA-827, StressGen, Canada). Visualization of antibody binding was carried out with ECL (Amersham Pharmacia Biotech).

3. Results

3.1. Enzyme activity of NS Cys mutants

In the NS protein, the cysteine at position 70 is one of the amino acid components of the C(X)PSR motif, part of the catalytic site and conserved within the sulfatase family. To investigate the importance of this residue in the NS protein, we generated by site-directed mutagenesis two mutated NS cDNAs in which the cysteine codon (Cys) was converted into a methionine (Met) or serine (Ser) codon, respectively. Wt and mutant expression plasmids were transiently co-transfected with β-galactosidase cDNA into COS cells by electroporation. After 48 h, the total cell extracts and the culture medium were analyzed for NS activity. The results are shown in Table 1: the untransfected COS cells (negative control) showed a residual activity of 4.9 nmol/17 h/mg; the COS cells transfected with the wt NS cDNA had an activity of 58.3 nmol/17 h/mg, i.e. a 12-fold increase. Cells expressing C70S and C70M mutants exhibited a drastic reduction in the catalytic activity, 7.8 and 8.6 nmol/17 h/mg, respectively. These results demonstrated that the mutations in the cysteine 70 residue resulted in a strong decrease in the catalytic activity of the NS enzyme, presumably as a consequence of an impairment of the normal function of the active site.

3.2. Western blot analysis

The presence of mutated NS polypeptides in transfected COS cell extracts was tested by immunoblot analysis using anti-NS antibody. In cells expressing the NS wt cDNA, two bands were visualized relating to the precursor (62 kDa) and the mature form (56 kDa) of the enzyme. Results comparable to wt were found in cells transfected with the C70S mutation (Fig. 1). In contrast, in cells transfected with C70M mutant cDNA, the precursor was significantly reduced, while the mature form was not detectable (Fig. 1), indicating that it was subjected to rapid degradation.

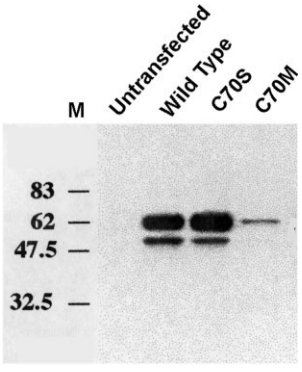

Fig. 1. Western blot analysis. 30 μg of total extracts of cells transfected with the NS wt and Cys mutant cDNA constructs was subjected to SDS–PAGE, followed by immunoblotting with anti-NS antiserum (1:500). M: molecular mass standards in kDa.

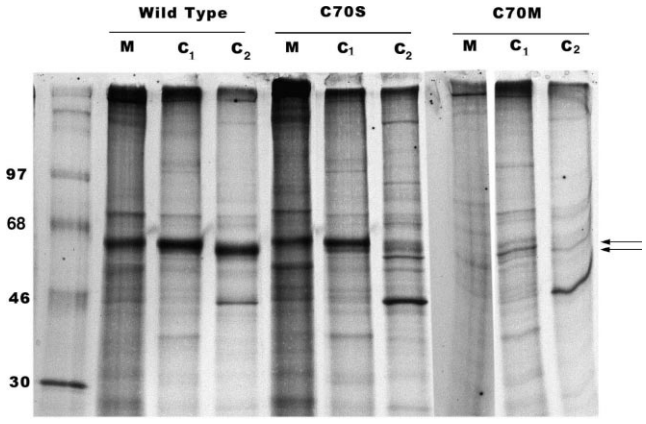

Fig. 2. Metabolic labeling of NS wt and Cys mutants. COS cells, transfected with the NS wt and Cys mutant cDNA constructs, were labeled for 2 h (pulse) and harvested after a subsequent 24 h chase period. NS polypeptides were immunoprecipitated from the media (M) or from cell pulse (C_1), and cell chase (C_2), separated by SDS–PAGE and detected by autoradiography. The arrows on the right indicate the NS 62 kDa precursor and the 56 kDa mature forms. Molecular mass markers are indicated on the left.

3.3. Metabolic labeling of normal and mutated NS protein

The biosynthetic pathway of wt and cysteine mutants was examined by pulse-chase labeling experiments; radioactivity incorporated into NS was determined after immunoprecipitation and separation by SDS–PAGE. The results are shown in Fig. 2. In COS cells transfected with wt NS, a precursor form of 62 kDa was evident after a 2 h pulse labeling that was converted into the 56 kDa mature lysosomal form or secreted

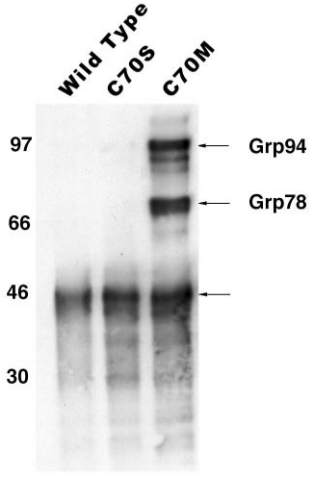

Fig. 3. Immunoblot recognizing BiP protein. Wt and Cys mutant COS cell extracts were immunoprecipitated with polyclonal anti-NS antiserum, then the denatured immunoprecipitates were resolved in 10% SDS gel and transferred onto the nitrocellulose membrane. BiP was detected by immunoblotting with an antibody recognizing Grp94 and Grp78; the antibody identifies, arrow on the right, also an aspecific protein of 40 kDa (see Section 2).

Table 1
NS activity of NS mutants transiently transfected in COS cells

Cell type	NS activity[a] (nmol/17 h/mg)	β-Galactosidase activity[a] (μmol/h/mg)	Normalized NS activity[b]
Untransfected	4.9 ± 2.0	8.5 ± 2.2	4.9
Wt	58.3 ± 3.3	84.2 ± 4.7	58.3
C70S	7.8 ± 0.9	72.5 ± 2.7	9.0
C70M	8.5 ± 1.0	71.6 ± 5.7	10.0

[a]Values are the mean ± S.D. of three independent experiments.
[b]By comparison of the β-galactosidase activity of the mutants with the β-galactosidase activity of the wt construct, to correct for transfection variability.

into the culture medium. C70S mutant cDNA, transfected in COS cells, encodes a protein with a similar precursor form of the enzyme (either in the medium or in the cells); however, a reduction was observed in the mature form to approximately 40% of the wt, as indicated by densitometric analysis of the bands (Fig. 2). Conversely, in cells expressing C70M cDNA, the NS protein was poorly synthesized with minute amounts of precursor form and no mature form within the cells; no precursor form was visible in the culture medium (Fig. 2).

3.4. Synthesis of C70S and C70M mRNAs in vitro

RT-PCR experiments were performed to examine if a transcript was present in the cells transfected with C70M mutant cDNA. To this purpose, total RNAs from COS7 cells transfected with wt, C70S and C70M cDNAs were reverse-transcribed using two oligonucleotides present on coding cDNA (see Section 2); a fragment of the expected size (137 bp) was amplified in all samples, demonstrating the presence of the respective RNAs (data not shown).

3.5. Replacement of the cysteine by a methionine causes association to BiP

To verify the hypothesized RER retention of C70M mutant protein, COS cells expressing wt NS, C70S or C70M proteins were lysed, immunoprecipitated with anti-NS antibody under non-denaturing conditions and the immunocomplexes, dissociated in denaturing lysis buffer, were loaded on a SDS–PAGE gel. A Western blot analysis was then performed with a monoclonal antibody recognizing BiP protein (Fig. 3). A significant amount of Grp94 and Grp78 co-immunoprecipitated with the C70M mutant, while a faint band representing Gpr78 was detected with C70S protein and no band was visible with the wt enzyme. These results indicate that the C70M is a misfolded protein that interacts with the molecular chaperone BiP.

4. Discussion

In this work, we performed site-directed mutagenesis on the conserved cysteine present in NS, the lysosomal enzyme whose deficiency causes MPS-IIIA [3]; using site-directed mutagenesis, we replaced cysteine 70 with two different amino acids: the serine residue with a hydrophilic hydroxyl group and the methionine containing a hydrophobic sterically encumbering ring. C70S and C70M mutant proteins were expressed in COS cells and analyzed for their catalytic properties and processing; both the cysteine replacements (C70S and C70M) had a drastic effect on the enzymatic activity, with loss of catalytic function (Table 1). Similar results have been reported for the Cys91 of *N*-acetylgalactosamine 4-sulfatase [19], the Cys84 of iduronate sulfatase [20], the Cys69 of arylsulfatase A [21], the Cys79 of *N*-acetylgalactosamine 6-sulfate sulfatase [22].

We show that the Cys70 residue of NS was critical also for enzyme processing and stability; by Western blot analysis it was possible to detect the 62 kDa precursor and the 56 kDa mature polypeptides in COS cells transfected either with wt or with C70S cDNAs, but only a minor amount of precursor form and no mature form in COS cells transfected with C70M cDNA. Furthermore, metabolic labeling experiments demonstrated a decreased amount of mature form for C70S protein; while a severely impaired biosynthesis of C70M mutant was evident, with neither the mature proteolytically processed protein nor any secreted enzyme. Studies performed in MSD fibroblasts showed that arylsulfatase A, arylsulfatase B and steroid sulfatase, although inactive, were normally processed [23]; results obtained from expression studies on the cysteine 84 mutant of iduronate sulfatase [20] showed that a C84A mutation had a drastic effect on processing, while C84T replacement produced a small amount of IDS inactive mature forms. Experiments performed on the cysteine 91 of *N*-acetylgalactosamine 4-sulfatase showed that the C91T replacement caused a lower level of intracellular protein [19]; however, a subsequent study revealed that the C91T was synthesized normally in the RER where it was apparently retained and degraded by the quality control system, but was not processed [24]. Also in this study, since RT-PCR analysis showed that the C70M mutant was correctly translated in vitro, the C70M protein seems to be synthesized but not proteolytically processed. The interaction of the C70M mutant with BiP, the molecular chaperone, suggests that a large part of this mutant is retained by the RER quality control system.

Previous studies have demonstrated that point mutations may directly affect the protein function or alter the correct folding and cause subsequent premature degradation of the mutant proteins within the RER; the resulting protein misfolding has been implicated in the pathogenesis of some genetic diseases [25,26]. The mechanism involved in the degradation of the mutant protein in lysosomal storage disorders has yet to be fully characterized, but recent evidence indicates the involvement of the degradation system in the RER quality control processes [24]; furthermore, protein conformation changes have been observed in the mutant 4-sulfatase protein in patient fibroblasts [19].

In conclusion, we confirm that the cysteine present in the active site of NS is essential for the catalytic function and have demonstrated that it is also important for the correct processing of this enzyme; moreover, the replacement of this residue may impair the folding and/or the stability of the protein.

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