Processing of normal lysosomal and mutant *N*-acetylgalactosamine 4-sulphatase: BiP (immunoglobulin heavy-chain binding protein) may interact with critical protein contact sites

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The lysosomal hydrolase *N*-acetylgalactosamine-4-sulphatase (4-sulphatase) is essential for the sequential degradation of the glycosaminoglycans, dermatan and chondroitin sulphate and, when deficient, causes the lysosomal storage disorder mucopolysaccharidosis type VI. The cysteine at codon 91 of human 4-sulphatase was identified previously as a key residue in the active site of the enzyme and was mutated by site-directed mutagenesis to produce a 4-sulphatase in which cysteine-91 was replaced by a threonine residue (C91T). The C91T mutation caused a loss of 4-sulphatase activity, a detectable protein conformational change and a lower level of intracellular 4-sulphatase protein [Brooks, Robertson, Bindloss, Litjens, Anson, Peters, Morris and Hopwood (1995) Biochem. J. **307**, 457–463]. In the present study, we report that C91T is synthesized normally in the endoplasmic reticulum as a 66 kDa glycosylated protein, which is very similar in size to wild-type 4-sulphatase. However, C91T neither underwent normal Golgi processing, shown by lack of modification to form mannose 6-phosphate residues on its oligosaccharide side chains, nor did it traffic to the lysosome to undergo normal endosomal–lysosomal proteolytic processing. Instead, C91T remained in an early biosynthetic compartment and was degraded. The molecular chaperone, immunoglobulin binding protein (BiP), was associated with newly-synthesized wild-type and mutant 4-sulphatase proteins for extended periods, but no direct evidence was found for involvement of BiP in the retention or degradation of the C91T protein. This suggested that prolonged association of mutant protein with BiP does not necessarily infer involvement of BiP in the quality control process, as previously implied in the literature. The predicted BiP binding sites on 4-sulphatase map to β-strands and α-helices, which are co-ordinated together in the folded molecule, indicating that BiP interacts with critical protein folding or contact sites on 4-sulphatase.

Key words: endoplasmic reticulum, molecular chaperones, mutant protein, protein conformation, protein processing.

INTRODUCTION

N-Acetylgalactosamine-4-sulphatase (4-sulphatase; EC 3.1.6.12) is a lysosomal enzyme which hydrolyses the 4-sulphate ester from non-reducing *N*-acetylgalactosamine residues of the glycosaminoglycans, dermatan sulphate and chondroitin sulphate. A 4-sulphatase deficiency in humans results in the lysosomal storage disorder, mucopolysaccharidosis type VI (MPS VI) or Maroteaux-Lamy syndrome, which is an autosomally-inherited genetic disease [1,2]. 4-Sulphatase and the associated disorder MPS VI have been used as a model system to study the processing of normal lysosomal protein and the handling of the mutant-gene product by the processing/folding machinery of the cells.

The rough endoplasmic reticulum (RER) is the site at which proteins are translated and translocated into the intracellular vacuole system. Within the lumen of the RER, molecular chaperones interact with nascent polypeptides to mediate protein folding, and early proteolytic and oligosaccharide side-chain processing occurs. A component of the RER system, termed 'quality control', ensures that proteins undergo correct folding, and improperly folded protein products are retained for subsequent degradation [3]. It is suggested that mutant-gene products are structurally altered and may not fold correctly, signalling the quality control system to retain and degrade the mutant protein. This removal process may occur in cases where the function of the protein has been only partially impaired, and results in a substantial contribution to pathophysiology [4]. This is exemplified in cystic fibrosis where the most common mutation, a deletion of phenylalanine-508 (ΔF508) in the cystic fibrosis transmembrane-conductance regulator (CFTR), causes misfolding and the ΔF508-CFTR protein is not trafficked to the cell surface [5]. The ΔF508-CFTR protein still forms a functional chloride channel [5] but appears to be retained in the RER by the molecular chaperone calnexin, which contributes directly to the disease state [6]. In cultured cells, conditions which promote protein folding, such as low temperature and glycerol treatment, can facilitate ΔF508-CFTR protein folding and result in trafficking of mutant but functional molecules to the cell surface [7].

4-Sulphatase is normally synthesized as a 66 kDa glycosylated precursor protein in the RER, which is targeted through and processed in the vacuolar network until it reaches the lysosome, where it is detected as a 57 kDa mature glycoprotein [8–11]. This 57 kDa mature form of 4-sulphatase is a monomer composed of disulphide-linked 43, 8 and 7 kDa subunits [12]. For human patients with MPS VI, there is a high degree of genetic heterogeneity amongst 4-sulphatase-mutant alleles [13], and reduced levels of 4-sulphatase protein have been observed in MPS VI fibroblasts ($< 5\%$ of normal controls; [14]). Protein conformational changes have been observed in the residual mutant 4-sulphatase protein detected in patient fibroblasts and in ex-

Abbreviations used: BiP, immunoglobulin binding protein; 4-sulphatase, *N*-acetylgalactosamine-4-sulphatase; C91, wild-type 4-sulphatase; C91T, 4-sulphatase mutation in which cysteine-91 is replaced with threonine; CFTR, cystic fibrosis transmembrane-conductance regulator; CHO, Chinese hamster ovary; RER, rough endoplasmic reticulum; MPS VI, mucopolysaccharidosis type VI.

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pression cell lines [11,14,15]. The retention and degradation of incorrectly folded 4-sulphatase protein in the RER could explain the lower levels of 4-sulphatase protein observed in MPS VI patient cells. Enzyme kinetic studies on residual 4-sulphatase protein indicate that, in most cases, if normal levels of protein were present in MPS VI patients then this would provide sufficient enzyme catalytic capacity to avert the clinical onset of the disorder [14,16].

A rigidly conserved region of sulphatases, recently identified as part of the active site of 4-sulphatase [17], has been used in the present work as a focus to study the folding/processing of normal and mutant 4-sulphatase. A site-directed mutation in 4-sulphatase cDNA, which converted the cysteine at codon 91 to threonine (C91T), was generated and expressed in Chinese hamster ovary (CHO) cells [11]. Cells expressing the C91T mutant produced low levels of conformationally altered 4-sulphatase protein when compared with cells expressing the wild-type 4-sulphatase (C91) [11]. In the present study, we report that C91T protein is synthesized normally in the RER but is not trafficked through and proteolytically processed in the vacuolar network, and is apparently retained and degraded by the RER quality control system. The resident RER molecular chaperone, immunoglobulin binding protein (BiP; GRP78), was shown to interact with both C91 and C91T. The role of BiP in the retention of C91T was investigated, but no direct evidence was found for the involvement of BiP in either the retention or degradation of mutant 4-sulphatase protein. Predicted BiP binding sites on 4-sulphatase mapped mainly to contact sites between α-helices and β-sheets and to other sites in the interior of the folded protein.

MATERIALS AND METHODS

Translation of 4-sulphatase cDNA *in vitro*

In vitro translation of radioactively-labelled 4-sulphatase was carried out using 4-sulphatase cDNA and a rabbit reticulocyte lysate translation system (either with or without the addition of dog pancreatic microsomes), according to the protocol supplied by the Promega Corporation (Sydney, N.S.W., Australia).

Cell culture and harvesting

CHO cells were transfected with either the gene for control C91 or C91T, and were cultured and harvested as described previously [11].

Biosynthetic labelling of CHO cells

Wild-type C91 and mutant C91T cell lines were labelled with [^{35}S]cysteine and [^{35}S]methionine protein-labelling mix (1175 Ci/mmol; NEN Research Products, Dupont, Melbourne, Vic., Australia). Cells (75 cm^2 culture flasks; Costar, NY, U.S.A.) were preincubated in 5 ml of cysteine- and methionine-free Dulbecco's modified Eagle medium (Life Sciences, Glen Waverley, Vic., Australia) containing 10 % (v/v) dialysed foetal-calf serum for 60 min and then labelled by adding 0.3 mCi of [^{35}S]cysteine and [^{35}S]methionine mix for 5 min or 2 h at 37 °C. Cells were washed three times with 5 ml of PBS and then either harvested [11], or chased by adding 5 ml of fresh Ham's F-12 medium (Gibco BRL, Glen Waverly, Vic., Australia) without label. Harvested cell pellets were resuspended in 100 μl of lysis buffer [10 mM Tris/HCl, pH 7.0/0.15 M NaCl/4 mM EDTA/1 % (v/v) Nonidet P40 containing protease inhibitors PMSF (0.2 mM), pepstatin A (1 μM) and leupeptin (1 μM)] at 4 °C. For immunoprecipitation with anti-BiP, 10 mM D-glucose and 5 units/ml of hexokinase were added to the lysis buffer. Cells were frozen and thawed six times, centrifuged at 12000 ***g*** for 5 min at 4 °C and the supernatant was collected as cell lysate. Labelling of cells with 0.5 mCi of [^{32}P]P_i (1 Ci/mmol; NEN Research Products) was carried out for 4 h at 37 °C, after preincubating for 60 min in phosphate-free RPMI 1640 medium (Gibco BRL) containing 10 % (v/v) dialysed foetal-calf serum.

Precipitation of protein from cell medium

Media from radioactively-labelled cells were each mixed with $(NH_4)_2SO_4$ at a concentration of 0.3 gm/ml overnight at 4 °C. The media precipitates were recovered by centrifugation (1000 ***g*** for 10 min at 4 °C), the pellets were resuspended in 1 ml of water and dialysed twice against 1 litre of 0.25 M NaCl/0.02 M Tris/HCl, pH 7.0, at 4 °C.

Immunoprecipitation

All procedures were carried out at 4 °C. Preimmune rabbit sera was coupled to Protein A–Sepharose (Amrad, Boronia, Vic., Australia) by mixing 1 ml of sera with 1 ml of packed Protein A–Sepharose overnight on a rotator, then recovered by centrifugation (800 ***g*** for 1 min) and washed three times with 10 ml of 0.25 M NaCl/0.02 M Tris/HCl, pH 7.0. Gelatin was coupled to AffiGel 10 [1 ml of gelatin (1 mg/ml) in 0.1 M $NaHCO_3$, pH 8.0, per ml of AffiGel 10] using the manufacturer's instructions (Bio-Rad, Richmond, CA, U.S.A.), then recovered by centrifugation (800 ***g*** for 1 min) and washed three times with 10 ml of 0.25 M NaCl/0.02 M Tris/HCl, pH 7.0. Radioactively-labelled cell lysates or precipitated cell media were precleared by mixing with 100 μl of preimmune rabbit sera–Protein A–Sepharose overnight. The precleared lysates were recovered by centrifugation (800 ***g*** for 1 min) and further adsorbed by mixing with 100 μl of gelatin–AffiGel 10 for 4 h. The supernatant was recovered by centrifugation (800 ***g*** for 1 min) and mixed with 5 μl of either polyclonal anti-4-sulphatase antibody (donated by CSL, Melbourne, Vic., Australia) or anti-BiP antibody [18] for immunoprecipitation (4 h). Antibody–antigen complexes were immobilized on to 100 μl Protein A–Sepharose by mixing overnight, then washed ten times with 10 ml of 0.02 M Tris/HCl, pH 7.0/0.15 M NaCl/4 mM EDTA/0.5 % (v/v) Nonidet P40 and washed a further three times with 10 ml of 0.25 M NaCl/0.02 M Tris/HCl, pH 7.0. The radioactively-labelled proteins were dissociated from the polyclonal antibody–Sepharose by boiling in reducing SDS/PAGE loading buffer for 5 min, separated by SDS/PAGE (10 % gel) [19] and, after incubation with the fluor Amplify (Amersham International, Little Chalfont, Bucks., U.K.), the bands were revealed by autoradiography.

In control precipitation experiments using either Protein A–Sepharose or Protein A–Sepharose plus non-immune rabbit sera, a 44 kDa protein was consistently observed and therefore identified as non-specific in the 4-sulphatase immunoprecipitates.

Dissociation of BiP from microsomal 4-sulphatase immunoprecipitates

Four 75-cm^2 flasks each of C91 and C91T cells were metabolically labelled with [^{35}S]cysteine and [^{35}S]methionine for 2 h at 37 °C. Cells from each cell line were harvested, pooled, washed with PBS (400 ***g*** for 5 min) and resuspended in 1.5 ml of homogenizing buffer [0.25 M sucrose/1 mM EDTA/10 mM Hepes/KOH, pH 7.5, containing the protease inhibitors PMSF (0.2 mM), pepstatin A (1 μM) and leupeptin (1 μM)]. Subcellular organelles were prepared by hyperbaric shock as described previously [20]. The post-nuclear supernatant was collected by centrifugation at 600 ***g*** for 10 min and the granular fraction was removed by centrifugation at 12000 ***g*** for 15 min. Microsomes were pelleted from the

remaining supernatant by ultra-centrifugation (100000 ***g*** for 60 min at 4 °C), then resuspended in 2 ml of lysis buffer [0.02 M Tris/HCl, pH 7.0/0.15 M NaCl/0.05 % (v/v) Nonidet P40] and freeze–thawed six times to prepare lysates. Microsomal lysates were immunoprecipitated with polyclonal anti-4-sulphatase antibody as described above, but without the gelatin-AffiGel preclearing step. Immunoprecipitates were divided into two portions; one half was eluted with reducing SDS/PAGE loading buffer and the other half with 1 ml of ATP elution buffer [0.02 M Tris/HCl, pH 7.0/0.15 M NaCl/0.05 % (v/v) Nonidet P40/6 mM $MgCl_2$/3 mM ATP]. The ATP-containing eluate was dialysed twice against 0.02 M Tris/HCl, pH 7.0, freeze–dried and resuspended in 100 μl of reducing SDS/PAGE loading buffer. After ATP elution, the remaining Protein A–Sepharose immunoprecipitates were recovered by boiling in 100 μl of reducing SDS/PAGE loading buffer.

Identification of BiP by Western blotting

Microsomal extracts were prepared from 10 flasks of C91-expressing CHO cells. Polyclonal anti-4-sulphatase antibody was cross-linked to Protein A–Sepharose with dimethylpimelimidate [21]. A 4-sulphatase immunoprecipitate was then prepared and separated by SDS/PAGE (10 % gel). Separated proteins were transferred to a PVDF membrane (Bio-Rad) by electroblotting for 1 h at 0.5 A in a Hoefer electrophoresis unit. The transfer membrane was incubated for 2 h in blocking buffer [1 % (w/v) dried milk/1 % (w/v) ovalbumin/1 M glycine]. After washing three times with PBS, the transfer membrane was incubated overnight at 4 °C with polyclonal anti-BiP antibody (Affinity Bioreagents, Golden, CO, U.S.A.) at a 1:250 dilution in PBS containing 1 % (w/v) ovalbumin. The transfer membrane was then washed three times with PBS and incubated for 1 h with a 1:1000 dilution of horseradish-peroxidase-conjugated sheep anti-rabbit immunoglobulin (Silenus Laboratories, Melbourne, Vic., Australia) in PBS containing 1 % (w/v) ovalbumin. Antibody reactivity was detected using enhanced chemiluminescence, according to the manufacturer's instructions (NEN Research Products).

Electron microscopy

CHO cells were fixed in 0.1 M cacodylate/5 mM $CaCl_2$, pH 7.2, containing 2 % (v/v) formaldehyde and 2 % (v/v) glutaraldehyde for 2–3 h, and were postfixed in 0.1 M cacodylate/5 mM $CaCl_2$, pH 7.2 containing 1 % (w/v) osmium tetroxide. Specimens were dehydrated in a graded series of ethanol and embedded in Spurr's low-viscosity epoxy resin (TAAB, Aldermaston, Berks., U.K.). Semi-thin (1 μm) survey sections were obtained using an ultramicrotome (Ultracut; Leica, Vienna, Austria) and stained with 1 % (w/v) Toluidine Blue in 1 % (w/v) disodium tetraborate. Ultrathin sections with silver interference colour (60–90 nm thick) were cut and mounted on 200-mesh square nickel grids (Guilder Grids, Grantham, Lincs., U.K.), and were stained with 4 % (w/v) uranyl acetate, followed by Reynolds lead citrate. The sections were then examined by transmission electron microscopy (Hitachi H-7000; Hitachi, Tokyo, Japan), operating at an accelerating voltage of 75 kV. For immunogold labelling, CHO cells were fixed in freshly prepared 4 % (w/v) formaldehyde and 0.5 % (v/v) glutaraldehyde in 0.1 M PBS, pH 7.4, for 1 h, followed by a 5 min wash in 0.1 M PBS, pH 7.4, to remove excess aldehyde. Specimens were dehydrated in a graded series of ethanol before being embedded in low-acid glycol methacrylate resin (LA-GMA; Structure Probe Inc., West Chester, PA, U.S.A.). Grids were preincubated with 0.01 M PBS, pH 7.4, containing 1 % (w/v) BSA (immunoglobulin free; Sigma). Sections were then incubated with rabbit anti-4-sulphatase polyclonal antibody diluted in PBS containing 1 % (w/v) BSA and 1 % (v/v) Tween 20 (Sigma), overnight at 4 °C. Grids were washed with PBS (three times for 5 min) and incubated with PBS containing 1 % (w/v) BSA for 5 min before incubation with Protein A–gold conjugate (10 nm, Batch 9537; Biocell Research Laboratories, Cardiff, U.K.) in the same buffer for 1 h. Grids were then washed with deionised water (three times for 5 min) and examined as described above.

Prediction of BiP binding sites

Potential BiP binding sites in the sequence of 4-sulphatase were identified using the BiP score computer program, as described previously [22,23], except that a recently updated scoring matrix was used (M.-J. Gething, unpublished work). This computer algorithm scores amino-acid sequences with a moving window of 7 residues, which corresponds to the apparent size of the polypeptide binding cleft on BiP. The new scoring matrix separates populations of binding and non-binding peptides with increased accuracy, and a cut off score of > 10 yields BiP binding peptides with greater than 80 % confidence. Potential BiP binding sites with scores > 10 were plotted on 4-sulphatase, using either the topological diagram described previously [17] or a space-fill molecular model (van der Waals radii plot) of 4-sulphatase, constructed using Netscape Chime™ [Molecular Designs Limited (MDL) Information Systems Inc.; http://www.mdli.com/wel.html].

RESULTS AND DISCUSSION

Synthesis and early processing of C91 and C91T 4-sulphatase

The synthesis and early processing of control C91 and mutant C91T were examined by a combination of translation *in vitro* and pulse–chase labelling experiments in CHO cell lines expressing human 4-sulphatase. C91 was shown to be synthesized in a cell-free translation system as a 54 kDa preprotein, which, in the presence of microsomes, was N-glycosylated to produce a 66 kDa precursor form (Figure 1). The additional 43 kDa protein ob-

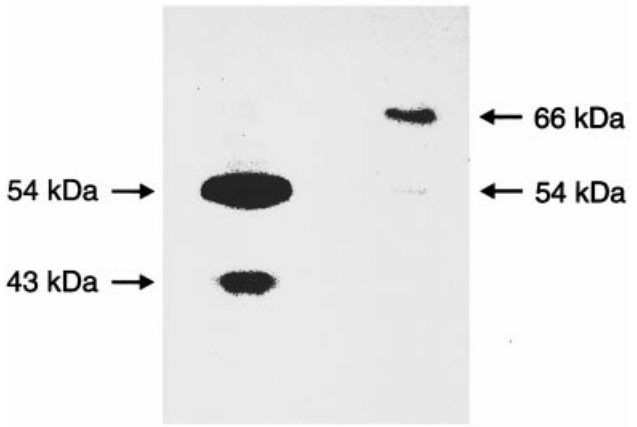

Figure 1 Translation of 4-sulphatase *in vitro*

In vitro translation either without microsomes (left lane) or with microsomes (right lane) after separation by SDS/PAGE and autoradiography. The molecular masses of the different forms of 4-sulphatase are marked. The presence of a 43 kDa form of 4-sulphatase was observed in 4-sulphatase translated in reticulocyte lysate but not in samples containing microsomes, and this may represent a proteolytically 'clipped' 4-sulphatase (i.e. in the absence of microsomes the protein would be exposed to proteases in the reticulocyte lysate).

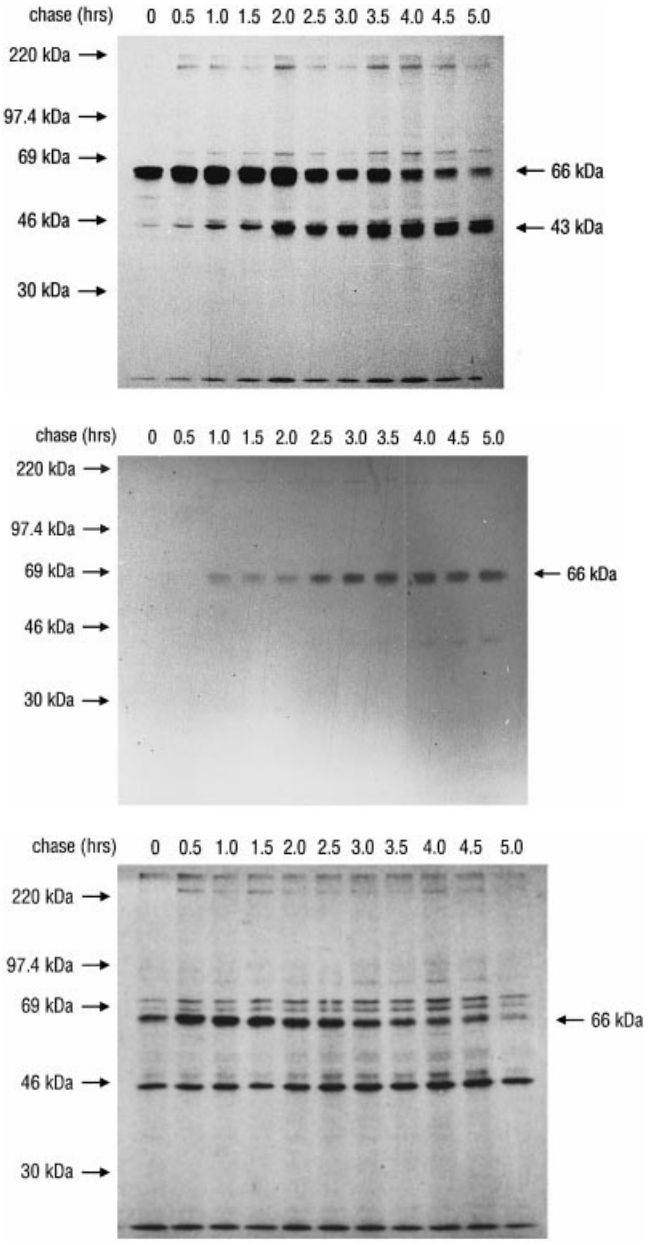

Figure 2 Processing of C91 and C91T

CHO cells expressing either C91 (top panel) or C91T (bottom panel) were pulse-labelled by incubation for 5 min in Dulbecco's modified Eagle's medium containing [^{35}S]methionine and [^{35}S]cysteine. Cells were then chased at 30 min intervals, up to 5 h, by incubating the cells at 37 °C in medium without label. After solubilization in 1% (v/v) Nonidet P40, cell lysates were immunoprecipitated with polyclonal anti-4-sulphatase antibody and the products were separated by SDS/PAGE and revealed by autoradiography (top and bottom panels). 4-Sulphatase protein in the cell medium was precipitated with $(NH_4)_2SO_4$, dialysed with 0.25 M NaCl/0.02 M Tris/HCl, pH 7.0, and immunoprecipitated with polyclonal anti-4-sulphatase antibody (middle panel). No 4-sulphatase was detected in the medium of CHO cells expressing C91T (results not shown). Precursor 4-sulphatase (66 kDa) and mature 4-sulphatase (43 kDa) are marked on the right and molecular-mass standards are marked on the left. Note: the 54 kDa band could be separated using non-reducing conditions, whereas the 8 kDa and 7 kDa forms of mature 4-sulphatase required specialized running conditions in order to separate them from the running front of the gel [12]. The 44 kDa protein shown in the top and bottom panel was detected also in control precipitations with either Protein A–Sepharose or Protein A–Sepharose plus non-immune rabbit sera (results not shown), and was therefore identified as non-specific in the 4-sulphatase immunoprecipitates.

served in the cell-free translation system without microsomes, was postulated to be a proteolytic product of the 54 kDa protein (i.e. in the absence of microsomes the protein would be exposed to proteases in the reticulocyte lysate) and not a second non-4-sulphatase translation product, as only one major glycoprotein was detected for translation in the presence of microsomes.

In CHO cells expressing 4-sulphatase, a 66 kDa glycosylated precursor form of C91 was synthesized in the RER (Figure 2). A 54 kDa processing intermediate observed in extracts of cells labelled for 5 min (Figure 2, top panel) probably represents a non-glycosylated form of 4-sulphatase corresponding to that seen in the *in vitro* translation experiments (Figure 1). When pulse-labelled cells were chased with non-radioactive medium for 1 h or more, C91 was either proteolytically processed to a 57 kDa, mature, lysosomal form [43 kDa, 7 kDa and 8 kDa subunits (the 7 and 8 kDa subunits needed specialized running conditions to separate them from the running front of the gel [12]); Figure 2, top panel] or secreted into the cell medium as a 66 kDa non-proteolytically processed form (Figure 2, middle panel).

In parallel pulse-label studies, C91T was detected in similar amounts and was of a similar molecular mass when compared with C91, indicating that the mutant protein was synthesized normally and appropriately glycosylated in the RER (Figure 2, bottom panel). Densitometric measurements (results not shown) indicated a similar half-life in the RER for the 66 kDa form of both C91 (2.5 h) and C91T (2 h). In fibroblast cells, wild-type 4-sulphatase has a much shorter half-life in the RER of 20–30 min ([10]; T. M. Bradford and D. A. Brooks, unpublished work), as measured by the disappearance of the 66 kDa glycosylated precursor and its associated proteolytic processing to the mature form (43, 7 and 8 kDa subunits), which occurs in the endosome/lysosome compartment. This difference in RER processing is presumably due to the over production of 4-sulphatase in CHO expression cell lines compared with fibroblasts (i.e. $\sim 10\ \mu g/mg$ in C91 CHO cells [11] compared with ~ 100 ng/mg in fibroblasts [14]). However, whereas the C91T protein was synthesized and glycosylated normally, its maturation was severely impaired, with neither mature proteolytically processed 4-sulphatase (e.g. the 43 kDa form; Figure 2, bottom panel) nor any secreted 4-sulphatase protein being detected in pulse–chase radioactively-labelled experiments (results not shown). The inability of the C91T polypetide to be processed to the mature form indicates that it does not reach the endosome/lysosome system. In addition, the failure to detect C91T protein in the cell medium confirmed that the protein had not reached the trans-Golgi and had then been secreted instead of being targeted to the lysosome. It therefore seemed likely that the C91T protein was retained and degraded within the RER.

Localization of mutant C91T to a pre-Golgi compartment

Localization of most or all of the mutant C91T protein within the RER compartment was confirmed by several approaches. First, immunogold labelling of 4-sulphatase, revealed by electron microscopy, lysosomal localization of C91 (Figure 3A, inset), but no lysosomal C91T could be detected in the endosomal/lysosomal compartment (Figure 3B, inset). There was no sign of distension of the RER in C91 and C91T cells (Figures 3A and 3B), due to either the increased synthesis of 4-sulphatase (compared with fibroblasts as described above) or the accumulation of misfolded 4-sulphatase. The sensitivity of the immuno-electron-microscopy technique was not sufficient to allow detection of 4-sulphatase in the RER of either C91 or C91T cells (Figure 3).

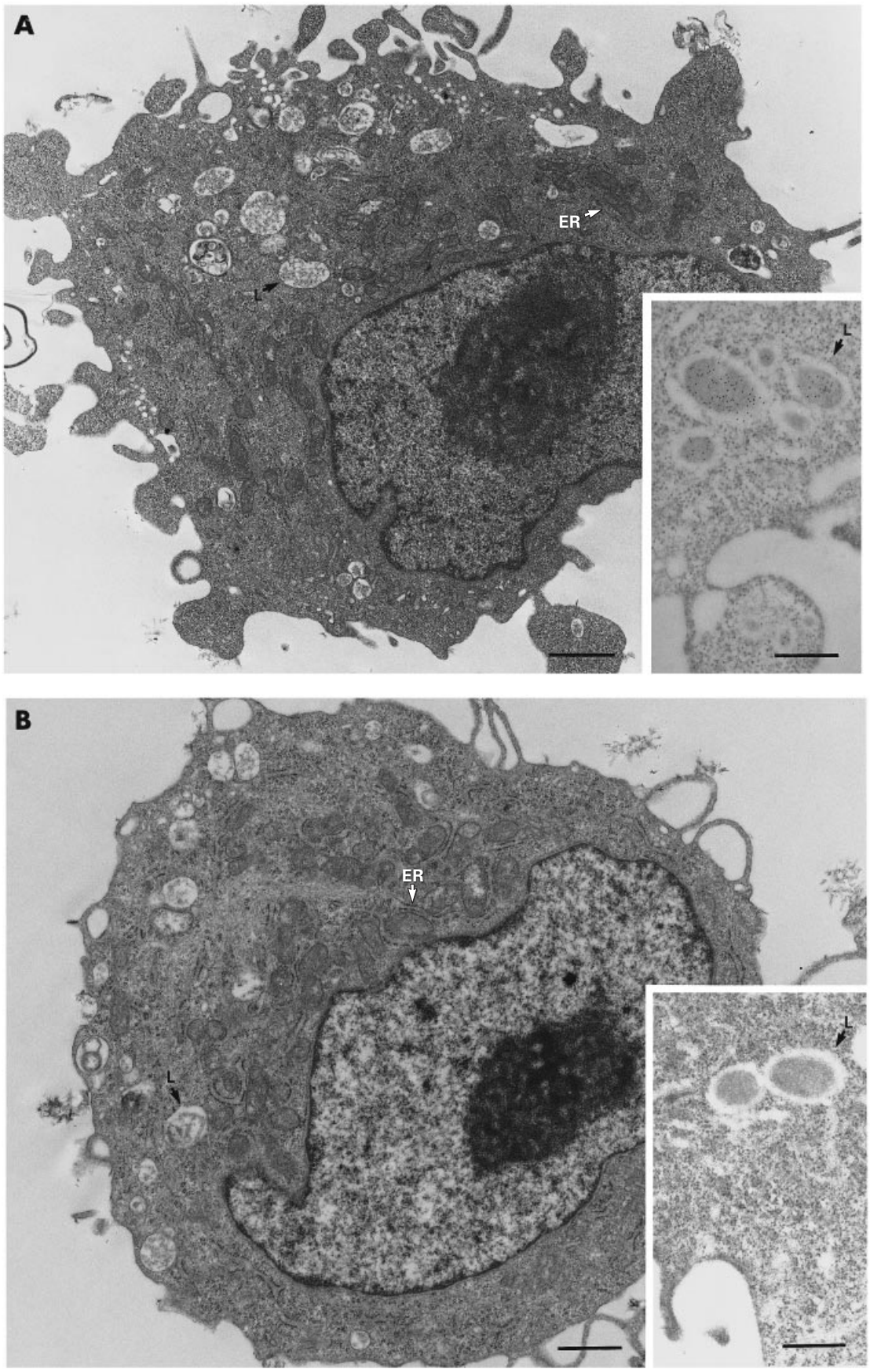

Figure 3 Localization of C91 and C91T in CHO cells

Ultrastructural morphology of CHO cells expressing (**A**) C91 and (**B**) C91T (× 20000 magnification). Insets show immunogold labelling of 4-sulphatase in lysosomes of these cells (× 30000 magnification). ER, endoplasmic reticulum; L, lysosome.

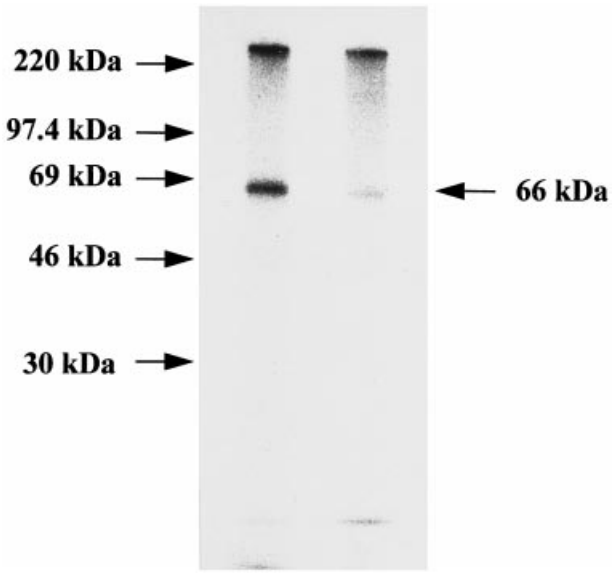

Figure 4 Phosphorylation of C91 and mutant C91T

CHO cells expressing either C91 (left lane) or mutant C91T (right lane) 4-sulphatase were incubated for 4 h in RPMI medium containing [^{32}P]P_i. Cells were lysed in Nonidet P40, immunoprecipitated with polyclonal anti-4-sulphatase antibody and the solubilized immune complex was separated on SDS/PAGE and visualized by autoradiography. The 66 kDa precursor 4-sulphatase is marked on the right and molecular-mass standards on the left.

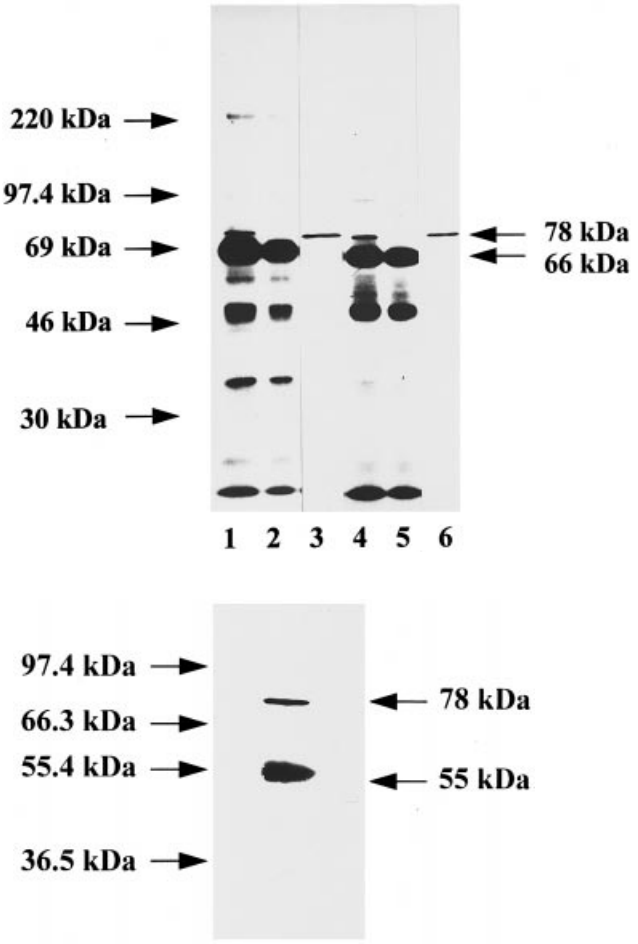

Figure 5 Dissociation of BiP from precursor 4-sulphatase with ATP-containing buffer

Upper panel: microsomal extracts from CHO cells expressing C91 (lanes 1–3) and C91T (lanes 4–6). Cells were metabolically labelled for 2 h with [^{35}S]methionine and [^{35}S]cysteine and immunoprecipitated with polyclonal anti-4-sulphatase antibody. Immunoprecipitates were eluted with either SDS/PAGE loading buffer (lanes 1 and 4) or ATP-containing buffer (lanes 3 and 6). Protein not eluted by ATP was subsequently eluted from the immunoprecipitates using SDS/PAGE loading buffer (lanes 2 and 5). The 78 kDa band was eluted from the immunoprecipitate with ATP buffer (lanes 3 and 6). Lower panel: identification of the 78 kDa band as BiP by Western blotting using polyclonal anti-BiP antibody. Some heavy-chain antibody from the immunoprecipitation was also seen on the Western blot because of reaction with the second antibody detection system (55 kDa). This 55 kDa band was also observed in a control where no primary antibody was added (results not shown), and represents the heavy chain of immunoglobulin which had leached out of the crosslinked Protein A–Sepharose. Precursor 4-sulphatase (66 kDa), BiP (78 kDa), polyclonal anti-4-sulphatase heavy chain (55 kDa) and molecular-mass standards are marked.

Endoglycosidase H sensitivity studies were performed on C91 and C91T in an attempt to determine whether the C91T mutant reached the Golgi apparatus. However, the mature lysosomal form of C91 retained endoglycosidase-H-sensitive N-linked oligosaccharides, preventing distinction between the Golgi processing of the mutant and control 4-sulphatase (T. M. Bradford and D. A. Brooks, unpublished work). As an alternative approach, the phosphorylation of C91T 4-sulphatase was investigated to determine if any of the mutant protein reached the cis-Golgi, since Golgi processing is required to generate the mannose 6-phosphate residues on N-linked oligosaccharides, which are essential for lysosomal targeting and the avoidance of the default secretory pathway. Cells expressing C91 or C91T were labelled with [^{32}P]P_i for 4 h and cell lysates were prepared and immunoprecipitated with anti-4-sulphatase polyclonal antibody. C91 was phosphorylated, but only minimal amounts of C91T were labelled (Figure 4), indicating that only trace amounts of mutant protein reached the Golgi apparatus. Taken together, these observations suggest that the great majority of the C91T protein is retained in the RER, a finding consistent with our previous report of the localization of C91T protein within crude microsomal cell fractions [11].

Studies on patients with lysosomal storage disorders have also implicated the RER in the retention of mutant lysosomal enzymes, including β-hexosaminidase in Tay-Sachs disease [24–26], α-*N*-acetylgalactosaminidase in Schindler's disease [27] and α-D-glucosidase in Pompe's disease [28]. In MPS VI patients, low levels of conformationally altered 4-sulphatase protein have been reported, corresponding to less than 5% of the catalytic capacity of normal human controls [14,16,20]. The normal level of synthesis of mutant 4-sulphatase protein observed in this and other studies [4,15,16] suggests that MPS VI patients have lower levels of 4-sulphatase because the protein is degraded. Quality control processes within the RER may remove and degrade mutant polypeptides which fail to fold correctly. In the case of most MPS VI patients this would contribute directly to the onset of pathology by removing partially functional protein. The mechanism of degradation of mutant protein in lysosomal storage disorders is yet to be fully characterized, but recent evidence indicates the involvement of a proteasomal degradation system in RER quality control processes [29–32]. It was concluded that the handling and fate of mutant C91T protein in cultured cells reflects the fate of mutant protein in these patient studies, with the conformationally altered C91T protein being retained and degraded in the RER.

Association of the molecular chaperone BiP with 4-sulphatase

The RER quality control process, which retains and signals the degradation of mutant lysosomal proteins, may be mediated by molecular chaperones, which are normally involved in protein folding in the RER. In the immunoprecipitation experiments described above (Figure 2, top panel, and Figure 2, bottom panel), some additional non-4-sulphatase proteins were observed that may correspond to co-immunoprecipitated molecular chaperones. To further characterize these proteins, CHO cells expressing either C91 or C91T were labelled with [^{35}S]cysteine and [^{35}S]methionine and microsomal fractions (enriched for RER) were prepared and immunoprecipitated with a polyclonal anti-4-sulphatase antibody (Figure 5). ATP buffer was used as a specific elutant to identify 4-sulphatase molecular chaperones requiring ATP for their action. A 78 kDa protein was eluted with 3 mM ATP from 4-sulphatase immunoprecipitates (Figure 5, upper panel; lanes 3 and 6). Identity of the 78 kDa protein as the RER resident heat-shock-protein 70 (HSP70) chaperone BiP was confirmed by immunoblotting with a specific polyclonal anti-BiP antibody (Figure 5, lower panel). Other proteins associating with immunoprecipitated precursor 4-sulphatase were not eluted with ATP-containing buffer and may represent proteins which react non-specifically either with the Protein A–Sepharose antibody complexes or with other molecular chaperones not released by ATP.

BiP has been shown to associate with mutant proteins in other disease states, including type I procollagen in osteogenesis imperfecta [33], glycosylation mutants of the α-subunit of β-hexosaminidase A in Tay-Sachs disease [34], mutant plasma serine protease precursor in Protein C deficiency [35] and mutant insulin receptors in patients with genetic forms of insulin resistance [36]. BiP has also been implicated in the RER retention of misfolded mutant insulin receptor and the elongated variant of Protein C. In these cases both BiP and the RER heat-shock-protein 90 (HSP90) chaperone Grp94 were more strongly associated with the mutant polypeptides than with the wild-type protein. The interaction of BiP in an ATP-dependent manner with both C91 and C91T warranted further investigation of the role of BiP in the folding of wild-type 4-sulphatase and of its possible role in the retention of the mutant protein.

Role of BiP in the retention of mutant 4-sulphatase protein

A prolonged association of BiP with 4-sulphatase was detected in both C91 and C91T cells. Immunoprecipitation of BiP from [^{35}S]cysteine- and [^{35}S]methionine-labelled CHO cells, expressing either C91 or C91T showed that similar amounts of wild-type and mutant 4-sulphatase were associated with BiP for chase times up to 90 min (Figure 6, upper panel). There was no apparent upregulation of BiP in C91T cells compared with C91 cells (Figure 6, upper panel). Subsequent immunoprecipitation of the extracts with anti-4-sulphatase did not detect any additional 4-sulphatase–BiP complexes (Figure 6, lower panel). Similar proportions of precursor 4-sulphatase were bound to BiP in each extract (Figure 6, upper panel).

In C91 cells the extended association of BiP with 4-sulphatase was presumably due to the relatively slow folding of the large amounts of 4-sulphatase that are produced by the overexpression vector. However, once folded the wild-type protein traffics through the vacuolar network to the endosomal/lysosomal compartment where it is proteolytically processed to the mature, active form. By contrast, C91T appears to be rapidly degraded without leaving the RER. Because BiP appeared to interact with the wild-type and mutant proteins to similar extents, we obtained no evidence for specific involvement of BiP in the degradation of

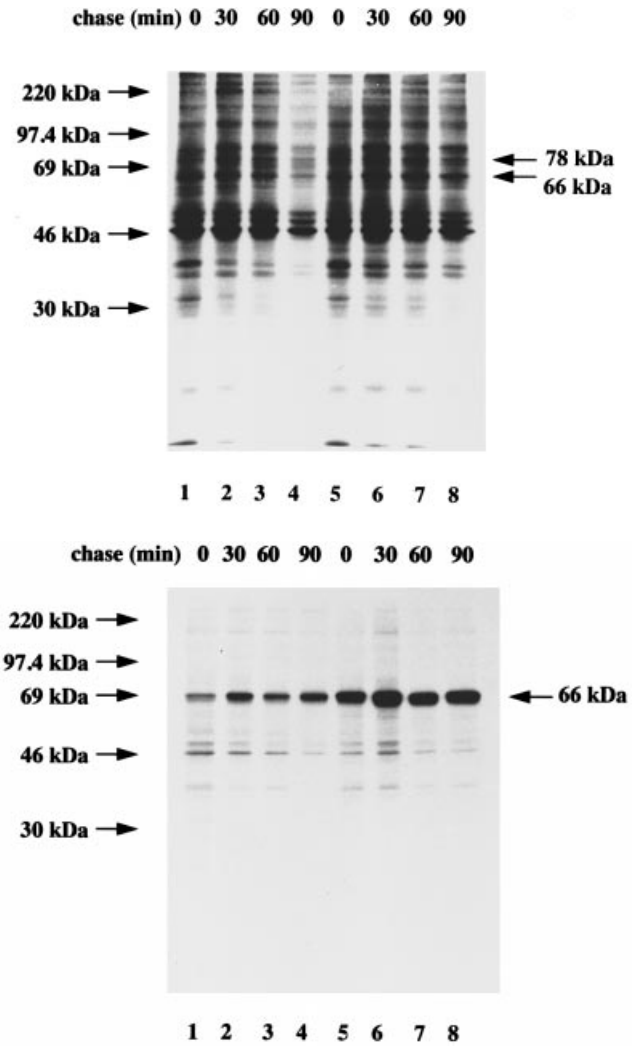

Figure 6 Association of 4-sulphatase with BiP

CHO cells expressing either C91 (lanes 1–4) or mutant C91T (lanes 5–8) were labelled by incubation in Dulbecco's modified Eagle's medium containing [^{35}S]methionine and [^{35}S]cysteine for 5 min. Cells were then washed and chased using unlabelled medium for either 30, 60 or 90 min. Cell lysates were prepared in the presence of 10 mM D-glucose and 5 units/ml hexokinase to deplete the amount of available ATP. Upper panel: cell lysates were immunoprecipitated with polyclonal anti-BiP antibody and were analysed by SDS/PAGE and autoradiography. Lower panel: unbound cell lysates, as described in the upper panel, were immunoprecipitated with anti-4-sulphatase antibody, separated on SDS/PAGE and visualized by autoradiography. BiP (78 kDa) and precursor 4-sulphatase (66 kDa) are marked on the right and molecular-mass standards are marked on the left.

C91T, implying that prolonged attachment to BiP is not sufficient alone to signal the degradation of 4-sulphatase. As noted above, the newly synthesized 66 kDa RER forms of C91 and C91T remain in the RER for similar times before embarking on their different processing pathways. It is not clear whether this is a coincidence or whether it reflects that the quality control system, which diverts the mutant protein to the degradative pathway, acts at a fairly late stage in the folding pathway.

Mapping of potential BiP binding sequences on 4-sulphatase and its role in 4-sulphatase folding

BiP binds to prefolded or malfolded polypeptides in the RER and recognizes linear heptapeptide sequences containing a pre-

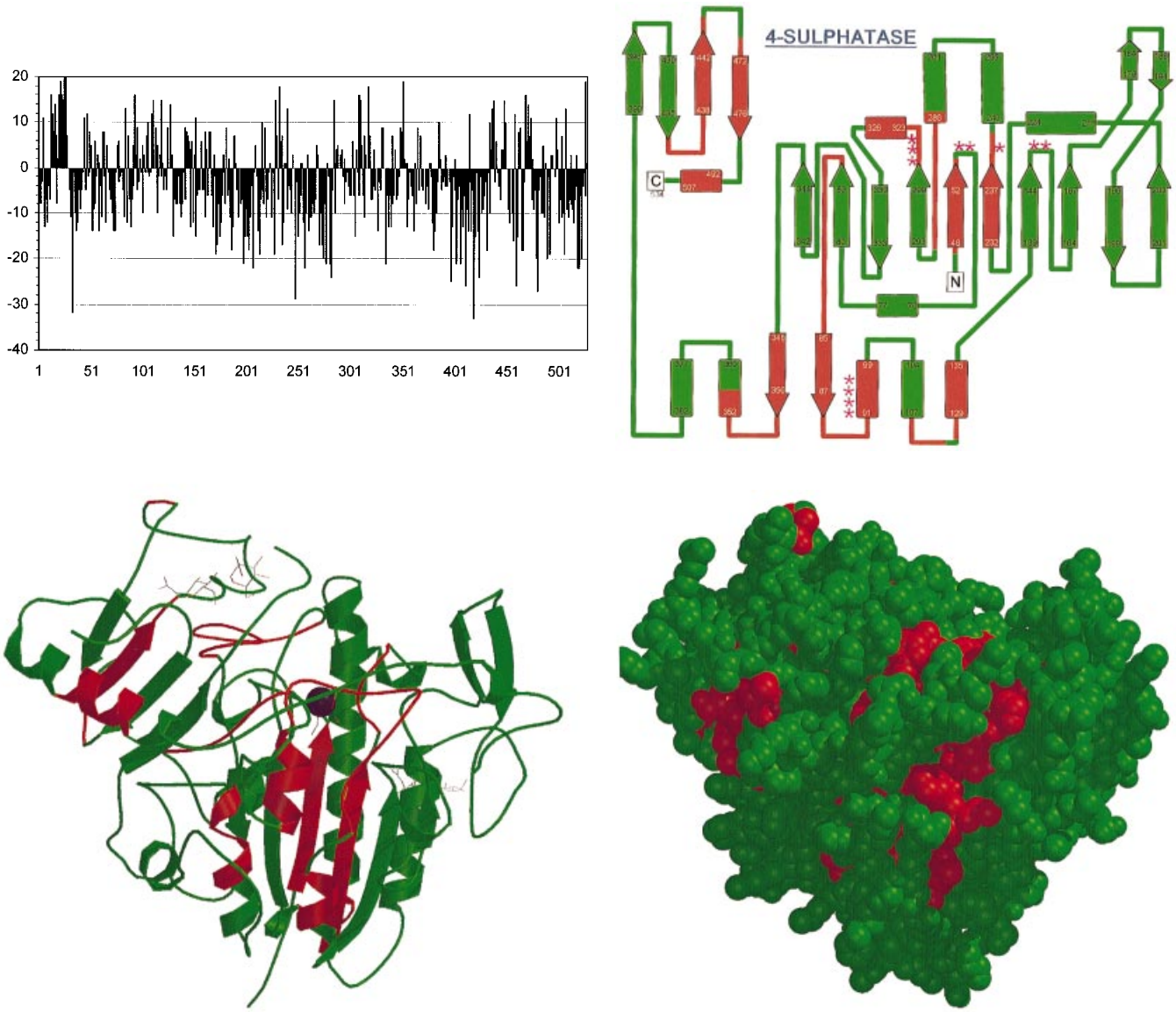

Figure 7 The mapping of potential BiP binding sequences on 4-sulphatase

Upper left panel: prediction of BiP binding sequences in the primary structure of 4-sulphatase. Overall scores for each of the overlapping heptapeptides in these sequences were calculated using the BiP Score program described by Blond-Elguindi et al. [22] using a modified scoring matrix (M.-J. Gething, unpublished work), and are plotted against the residue number of the first amino acid of each heptapeptide. Upper right panel: predicted BiP binding sites (red) were plotted on a topological map of 4-sulphatase (green), where arrows represent β-pleated sheets and rounded rectangles represent α-helices. Active-site residues are marked with a magenta asterix. Lower left panel: BiP sites (red) plotted on a ribbon diagram of folded 4-sulphatase (green) constructed using Netscape Chime™ [Molecular Designs Limited (MDL) Information Systems Inc.; http://www.mdli.com/wel.html]. Lower right panel: BiP sites (red) plotted on a molecular drawing of 4-sulphatase (green), which represents a space filled molecular model (van der Waals radii plot) of 4-sulphatase, constructed using Netscape Chime™ [Molecular Designs Limited (MDL) Information Systems Inc.; http://www.mdli.com/wel.html].

ponderance of bulky aromatic and hydrophobic residues, whose side chains would normally be buried in the interior of a folded protein [22,37]. We took advantage of the BiP Score computer program [22], which previously has been used successfully to predict BiP binding sites within the sequences of several different secretory proteins [38,39]. Figure 7 (upper left panel) shows the result of the scoring procedure. The scores range from -32 to $+20$, but only a small proportion of the heptapeptides have scores > 10, indicating that they have a very high probability ($>$ 80 %) of binding to BiP. A cluster of about 20 peptides with high positive scores are located at the N-terminus of the protein and correspond to the signal sequence. The presence of multiple peptides with high BiP scores within signal peptides has been noted previously [22]. The other peptides with high positive scores are distributed throughout the sequence of 4-sulphatase. The availability of the crystal structure of 4-sulphatase [17] allowed us to map the potential BiP binding sites (scores > 10) on to a molecular model of the folded protein. 4-Sulphatase had 11 regions in which one or more potential BiP binding sites were identified (Figure 7, upper right panel). Figure 7 (lower left panel) shows how these potential binding sites are located in sequences that form α-helical and β-sheet secondary structural

elements of 4-sulphatase. Some of the binding sites co-ordinate to form the active-site cleft in the folded molecule and others are in segments that join structural elements. Interestingly, several of the active-site residues [17] are either very close to or included within potential BiP binding sites. These sites may be available for binding of BiP in the unfolded molecule but, once folded, the active site residues (which, with the exception of the catalytic cysteine-91, are either polar or charged) form a hydrophilic substrate-binding cleft, whereas the side chains of the neighbouring hydrophobic residues that are recognized by BiP are buried in the interior of the protein (Figure 7, lower right panel). One predicted BiP binding site was located on the surface of 4-sulphatase and the hydrophobic side chains that form the BiP-recognition motif were exposed, suggesting that the binding site could be maintained in the folded molecule (Figure 7, lower right panel). The role of BiP during the folding of 4-sulphatase *in vivo* is likely to be mainly to protect hydrophobic sequences and surfaces from aggregation prior to their burial within the folded molecule, as has been suggested previously for other proteins that enter and traverse the secretory pathway [22,23,39,40]. BiP could also help ensure the correct folding of the active site of 4-sulphatase by co-ordinating the contacts between the secondary elements that form the binding cleft. Knarr et al. [23] reported that BiP bound sites on the immunoglobulin nascent heavy chain that are contact sites between the heavy and light chains in the folded molecule. This is similar to the contact domains identified for 4-sulphatase and may suggest a common role for BiP in stabilizing these interactions.

Conclusions

The processing and transport to the lysosome of mutant C91T 4-sulphatase was impaired. The previously observed conformational changes in the C91T protein appear to initiate quality control processes in the RER, which prevent normal trafficking along the secretory pathway and result in degradation of the mutant polypeptide. This is consistent with analyses in MPS VI patients, where only low levels of conformationally altered 4-sulphatase can be detected. However, in most cases, the mutations found in the disease do not completely abrogate the activity of this residual 4-sulphatase protein. The RER quality control process therefore contributes directly to the development of pathophysiology in these patients, by removing the mutated, conformationally altered but functional 4-sulphatase protein. Whereas BiP is likely to play a critical role in the folding of 4-sulphatase, no evidence could be obtained for its direct involvement in the subsequent degradation of C91T 4-sulphatase. Most BiP sites appear to be internalized to form part of the globular core of folded 4-sulphatase, but one predicted site remained exposed at the surface. This site may have a role in attachment to BiP/heat shock protein 70 later in the endocytic network (i.e. postRER).

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