Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2009; 34 (3) ; 160–166.

Research Article [Araştırma Makalesi]

Yayın tarihi 25 Eylül, 2009 © TurkJBiochem.com

[Published online 25 September, 2009]

Inhibition of Endoplasmic Reticulum Associated Degradation of Mutant Pro-β Subunits of β-Hexosaminidase by Kifunensine and Lactacystin

[β-Heksozaminidaz Mutant Öncül-β Altbirimlerin Endoplazmik Retikulum İlişkili Yıkımının Kifunensin ve Laktasistin ile İnhibisyonu]

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Registered: 22 July 2009; Accepted: 3 August 2009
[Kayıt tarihi: 22 Temmuz 2009; Kabul tarihi: 3 Ağustos 2009]

ABSTRACT

Objectives: We investigated the stabilizing effects of inhibiting Endoplasmic Reticulum Associated Degradation using kifunensine and lactacystin on heterologous expression of two mutant pro-forms of β-subunit of β-hexosaminidase, 12 bp deletion and D208N missense mutations.

Methods: The Δ12 bp and D208N mutations were introduced into β-subunit cDNA of hexosaminidase by site-directed mutagenesis. Mutant (Δ12 bp) or wild-type β-subunit cDNAs were transfected to COS-1 cells. At 5th h of transfection, 200 μM kifunensine or 1.5 μM lactacystin were added to the medium. Fourty-eight hours after lysed cells were subjected to Western Blot analysis. D208N stably expressing CHO cells were grown with or without lactacystin or kifunensine in the presence of radiolabeled with ^{35}S-Met and ^{35}S-Cys and chased with cold media for different durations. Labeled hexosaminidase was immunoprecipitated with rabbit anti-human hexosaminidase A antibody, resolved by SDS-PAGE and visualized by autoradiography.

Results: Lactacystin or kifunensine treatment of cells expressing either of the two mutants resulted in increased levels of mutant pro-β chain. A corresponding increase in enzymatic activity was not observed in any of the mutants.

Conclusion: Both mutant forms of β-subunit are degraded through mannosidase/EDEM-1 ubiquitin/proteasome pathway. This is consistent with the model whereby β-subunit of hexosaminidase is a substrate for the calnexin/calreticulin folding pathway in the endoplasmic reticulum. Although inhibiting either of these systems results in a increased steady-state level of mutant protein, neither leads to increased enzymatic activity.

Key words: kifunensine, lactacystin, hexosaminidase B, endoplasmic reticulum associated degradation, Turkish Tay-Sachs mutation

ÖZET

Amaç: Kifunensin ve laktasistinin, β-heksozaminidaz β-altbiriminin iki mutant öncülşeklinin (12 bç'lik delesyon ve D208N yanlış anlamlı mutasyon) heterolog ekspresyonları üzerinde, Endoplazmik Retikulum ile İlişkili Yıkımın inhibisyonunu stabilize edici etkileri araştırıldı.

Gereç ve yöntemler: Yönlendirilmiş mutagenez ile Δ12 bç ve D208N mutasyonları, heksozaminidazın β-altbirim cDNA'sına yerleştirildi. Mutant (Δ12 bç) veya normal β-altbirim cDNA'ları, COS-1 hücrelerine transfekte edildi. Transfeksiyonun 5. saatinde, 200 μM kifunensin veya 1.5 μM laktasistin kültür ortamına eklendi. Transfeksiyondan 48 saat sonra, hücre lizatları Western Blot analizine uygulandı. Kalıcı şekilde D208N eksprese eden CHO hücreleri, ^{35}S-Met ve ^{35}S-Cys ile radyoaktif işaretlenmiş ve farklı zamanlarda takip edilmiş soğuk besi yerinde, laktasistin veya kifunensin varlığında ve yokluğunda büyütüldü. İşaretli heksozaminidaz, tavşan anti-insan heksozaminidaz A antikoru ile çöktürüldü; SDS-PAGE ile çözüldü ve otoradyografi ile gözlendi.

Bulgular: Laktasistin veya kifunensin ile muamele edilmiş her iki mutantı eksprese eden hücrelerde, mutant öncül-β zincir proteini artmış düzeyde bulundu. Herhangi bir mutantta benzer şekilde artmış enzim aktivitesi gözlenmedi.

Sonuçlar: β-altbirimin her iki mutant şekli, mannozidaz/EDEM-1 Ubikütin/Proteozom yolunu kullanarak yıkılmaktadır. Sonucumuz, heksozaminidaz β-altbiriminin, endoplazmik retikulumdaki kalneksin/kalretikülin katlanma yolu için substrat olduğunu belirten model ile uyuşmaktadır. Bununla birlikte, sistemlerin herhangi birinin inhibisyonu, mutant proteinin kararlı düzeyinde artışa neden olurken enzimatik aktivite artışına yol açmamaktadır.

Anahtar kelimeler: kifunensin, laktasistin, heksozaminidaz B, endoplazmik retikulum ilişkili yıkım, Türk Tay-Sachs mutasyonu

Introduction

Protein misfolding has been recognized as an important cause of protein deficiency in various inherited disorders (1-3). To maintain the integrity of proteins synthesized in the endoplasmic reticulum (ER), cells have evolved an efficient ER 'quality control' system (ERQC). This system has been mathematically modeled by breaking it into three subpathways; termed the Endoplasmic Reticulum Associated (ERA) Degradation (ERAD), ERA-folding and ERA-transport pathways (4) The ERQC assures that only properly folded and assembled proteins are transported to the Golgi for further maturation. Improperly folded proteins are retained in the ER for either subsequent export to the cytosol and degradation by the proteosome (5,6), or removal from the ER through the macroautophagy–lysosome system (7). Folding of glycoproteins in the ER often involves the calnexin/calreticulin system (the best characterized of the ER associated folding pathways), which includes glucosidases I and II, and the ER folding sensor UDP-glucose–glycoprotein glucosyltransferase. Terminally misfolded proteins become substrates for ER α-mannosidases and subsequently ligands for the ER degradation enhancing α-mannosidase I-like protein (EDEM), which then induces the retro translocation and degradation process of ERAD (8-11). Removal of a mannose residue from Man9 N-linked oligosaccharides by ER α-mannosidase I is a critical step for preventing misfolded proteins from reentering the ER folding pathway and serves as a signal for retrotranslocation and proteosomal degradation (12). Kifunensine is a selective inhibitor of the ER α-mannosidase I (13). Lactacystin is a cell permeable irreversible proteasome inhibitor. These compounds have been used to inhibit ERAD and increase the levels of misfolded proteins in the ER. It has been suggested that increasing the concentration of mutant protein in the ER also promotes its ability to fold and exit the ER (14).

Protein misfolding due to missense mutations or inframe deletions, can lead to β-hexosaminidase (Hex) A deficiency and GM2 gangliosidosis. GM2 gangliosidosis can result from mutations in either of the evolutionarily related *HEXA* or *HEXB* genes encoding the α subunit (Tay-Sachs disease) or β (Sandhoff disease) subunit of Hex (15). One of these mutations is the 12 bp deletion (Δ12 bp, 1096–1107 or 1098–1108 or 1099–1109) in exon 10 of *HEXA* gene (16,17). Previous studies have examined the biological effects of this mutation in the original α-subunit and by introducing it into the the aligned position of the more stable β-subunit of Hex. These studies showed that regardless of the subunit that contains the mutation, it remained in its pro-ER form without further processing to its mature lysosomal form (18,19). The other mutation, D208N, is near the active site of the β subunit and is conserved in 15 different species of Hex. Hou et al. showed that D208 is very important in the initial folding and/or stability of the pro β-chain and is unlikely to be involved in substrate binding or catalysis (20, 21). Small molecule compounds known as pharmacological chaperones (PC), which function either as agonists or competitive inhibitors, have been used to rescue several misfolded proteins from ERAD (6,21-23) and represent a new emerging threrapeutic approach. PC's are agents of enzyme enhancement therapy (EET) that rescue a proportion of the misfolded proteins by increasing the stability of the properly folded form of the protein in ER. Only 5–10% of normal enzyme levels is needed to reduce and⁄or prevent substrate accumulation in Tay-Sachs disease (24,25). This surprisingly low "critical threshold" of activity indicates that even small increases in patients' residual Hex A levels can dramatically modify their clinical phenotype. Although both the α and β subunits of Hex A are glycoproteins, it has not been demonstrated that these proteins are substrates for the calnexin/calreticulin system.

In this study, we assess the involvement of the calnexin/calreticulin pathway and the ubiquitin-proteosome system in the degradation of two mutant forms of the β subunit of Hex. A Δ12 bp mutation was engineered into the β-cDNA (1267-1278), which we have previously characterized, that aligns with a known mutation in the α-subunit associated with infantile Tay-Sachs disease (17-19). Although it is not associated with GM2 gangliosidosis, the D208N mutation was produced to probe the function of this conserved residue. When expressed in tranfected CHO cells only the pro β, ER-form of the D208N subunit was detected (20). Indirect immunofluorescence microscopy using an anti-human Hex B antibody demonstrated that this mutant protein is trapped in the ER (21).

Materials and Methods

Site directed mutagenesis, cloning and transformation

The 12 bp deletion mutation was introduced in nucleotide positions 1267-1278 of human β-subunit cDNA of Hex by two-step overlap extension PCR (19). Mutated PCR product was tagged with deoxyadenosines and cloned into pcDNA2.1-TOPO according to manufacturer's instructions (Invitrogen, USA). Cloning was performed by coupling of deoxyadenosines of PCR product with deoxytimidines in PCR2.1-TOPO. Chemical transformation was conducted to DH5α competent cells (Invitrogen, USA). Clones were isolated by QIAprep Kit (Qiagen, Canada). The 12 bp deletion mutation, 5' to 3' orientation and the exclusion of extraneous mutations was confirmed in all candidate mutant β-cDNAs by sequencing in their entirety (ACGT Corp, Toronto, ON, Canada). Interestingly, 12 bp deletion mutation eradicates the only *Bam*H1 restriction site in β-cDNA. Thus, we were able to check the mutation in all candidate β-cDNAs by restriction digestions with *Bam*H1 for 3 hrs at 27 ^{0}C, followed by agarose gel electrophoresis to

analyze the fragments (Figure 1). One of the confirmed mutated clones was selected and a double restriction digestion was performed with *Bam*HI and *Xho*I to obtain mutated β-cDNA with cohesive ends. The mutant β-cDNA was cloned into *Bam*HI-*Xho*I cohesive cloning sites of the pIRES2-EGFP mammalian expression vector by ligation. Ligation was performed overnight at 4 ^{0}C by T4 ligase (Roche, Germany). Mutant pIRES2-EGFP constructs were transformed into competent DH5α cells and clones were verified by DNA sequencing of the entire β-cDNA. Construction of βHex D208N expression plasmid and generation of stably expressing CHO cell lines has been previously described (21).

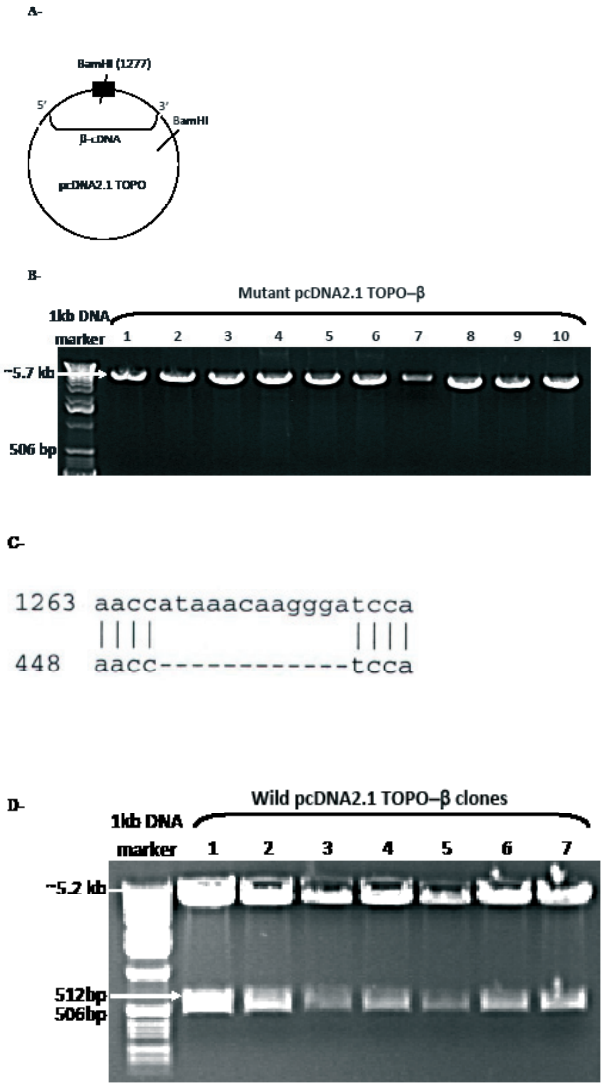

Figure 1. Confirmation of the presence of the Δ12 bp mutation in the β-cDNA insert of pcDNA2.1-TOPO clones. A) pcDNA2.1-TOPO plasmid carrying the β-cDNA. There is one restriction site for BamHI in the sequence of pcDNA2.1-TOPO and one in the wild-type β-cDNA. The Δ12 bp mutation erases the BamHI restriction site in β-cDNA. B) All of the 10 clones isolated were lacked the wild-type BamH1 site producing the single 5.7 kb band. C) Confirmation of the Δ12 bp mutation in mutant pcDNA2.1-TOPO clone by sequencing. The upper row of neucleotides is the normal β-cDNA sequence. D) The isolated 7 clones which carry the wild-type β-cDNAs. Both the plasmid and wild β-cDNA were confirmed to have the cut by intact BamHI site which results in a 5.2 Kb and a 506 bp band.

Cell culture and transfection

COS-1 cells were grown in α-MEM (minimal essential medium) with 10% FCS (fetal calf serum), penicillin and streptomycin at 37 ^{0}C in 5% CO_2. Cells (40% confluent) were transfected with 10 mg of pIRES2-EGFP plasmid, containing a wild-type or mutant β-cDNA insert, and 10 μg of pIRES2-EGFP (mock transfected) using Polyfect reagent (Qiagen, Canada) according to the manufacturer's protocol. After 48 hrs, the transiently transfected cells were trypsinized and harvested.

Treatment with ERAD inhibitors

COS-1 cells transiently transfected with mutant β-cDNA were treated with a final concentration of 1.5 μM lactacystin (Sigma, Canada) in 60 mm cell dishes, at the 5th hr after transfection. Another set of mutant β-cDNA transiently transfected COS-1 cells were treated with a final concentration of 200 μM kifunensine (Calbiochem, Canada) in 60 mm cell dishes, at the same time as in the lactacystin-treatment. Cells were collected after 48 hrs. Cell lysates were prepared in 10mmol/L sodium phosphate buffer, pH 6.0, and 5% glycerol by freezing-thawing and treatment with 1% (v/v) P-2714 protease inhibitor cocktail (Calbiochem, Canada) in 50% PBS-Triton X100. Then cell lysates were assayed for total protein, Hex and β-glucuronidase activity levels.

Western Blot analysis

Samples of transfected and mock transfected COS-1 cell lysates, carrying equal β-glucuronidase activities (60 nmol/h per mg protein), were resolved by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE) on 12.5% gel by the Laemmli using Bio-Rad mini-gel system (26). Proteins were transferred to nitrocellulose overnight at 4 ^{0}C. The filter was blocked in 5% powdered skim milk in TBST (10 mM Tris base, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 4 hrs followed by overnight incubation with 1/800 dilution (1% skim milk in TBST) of rabbit anti-human Hex B (laboratory of D.J.M.) (27). Nitrocellulose was washed four times with (1% skim milk in TBST) and was incubated with a 1/10 000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno Research, PA, USA) in 1% skim milk in TBST for 1 hr. Bands were visualized using chemiluminescent substrate as described in the Amersham ECLsystem (Amersham, Bucks, UK) (28).

Enzyme activities

Protein from cell lysates was quantified according to the Bradford method (Bio-Rad, CA, USA) (29). β-glucuronidase and Hex activities from cell

lysates were determined using the fluorogenic substrates 4-methylumbelliferyl β-glucuronide and 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG) (30,31). Specific Hex and β-glucuronidase activities were determined as nmol/h per mg total protein.

Pulse chase analysis

Stably expressing βHex D208N CHO cells in 60mm dishes grown to 50% confluence were starved for 1.5-2.5 hrs by incubation in Met- and Cys-free alpha-MEM (Invitrogen,CA,USA) media containing 10% dialyzed fetal calf serum (Invitrogen, CA,USA). For radiolabelling cells were pulse labelled with 100 µCi ^{35}S-Met, ^{35}S-Cys (Trans-^{35}S label™, MP biomedicals, OH, USA) for 30 min in 1 mL of media at 37° C. Subsequently, labeling media was removed, cells were washed twice with complete media (alpha-MEM with 10% Fetal calf serum) and followed by a chase period of variable duration in complete media. Kifunensine (Toronto Research Chemicals, ON, Canada) treated cells were incubated with the drug (200 µM final) during the starvation, pulse labeling and chase periods. Lactacystin (Calbiochem, CA, USA) treated cells were incubated with the drug (20 µM final) only during the chase period. For immunoprecipitation, cells were washed twice with PBS, scraped into 1 mL PBS, pelleted by centrifugation and lysed in 1mL RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) containing leupeptin (10 µg/ml), phenylmethanesulphonylfluoride (0.5 mM PMSF) and iodoacetamide (10 mM) by incubation at 4° C for 20 minutes followed by centrifugation in a microfuge for 15 minutes at maximum speed. Lysate was incubated with rabbit polyclonal Ab against human placental HexA (1 µg/mL, IgG fraction) followed by addition of Gamma bind Sepharose beads (10 µL packed volume) for 1 hr at 4° C. Beads were washed twice with RIPA buffer and bound protein eluted by incubation in RIPA buffer containing 33 µM DTT. Proteins were resolved by SDS-PAGE on a 12% gel and visualized by autoradiography.

Results

Site directed mutagenesis

Mutant β-cDNA construct was generated by site-directed mutagenesis. Initial screening for the presence of the mutant was through the loss of a specific BamH1 site in the insert (Figure 1). The presence of the single-target mutation was then confirmed by DNA sequencing of the entire insert. Before transfection, pIRES2-EGFP plasmids were confirmed to be carrying either wild type β-cDNA or mutant β-cDNA inserts (Figure 2).

Western Blot expression

Extracts from cells tranfected with the wild-type vector contained both forms of the human β-protein, the pro-β

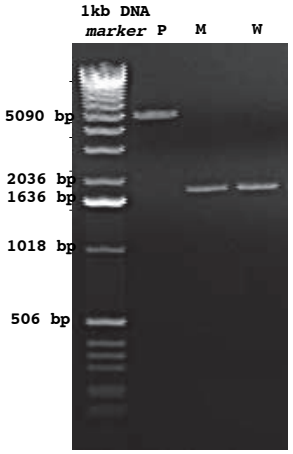

Figure 2. Confirmation of the insert β-cDNA inserts in the expression vector before transfection. P, pIRES2-EGFP plasmid without β-cDNAs. M, pIRES2-EGFP plasmid carrying mutant β-cDNA. W, pIRES2-EGFP plasmid carrying wild β-cDNA.

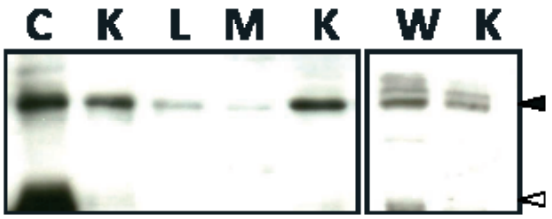

Figure 3. Steady state levels of WT or mutant Δ12bp β -protein in the absence or presence of kifunensine or lactacystin. Western Blot analysis showing the β -protein expression levels. Lysates, prepared from treated transfected COS cells, were subjected to western blot analysis. Blots were probed with a rabbit antibody against human Hex A (cross-reacts with both α- and β -subunits) and binding visualized using a secondary antibody against IgG conjugated with peroxidase. Labelled lanes correspond to; (C) COS cells transfected with pEFneo carrying wild-type β-cDNA, (K) COS cells transfected with pIRES2-EGFP carrying mutant β-cDNA and treated with kifunensin, pro β band is stronger, (L) COS cells transfected with pIRES2-EGFP carrying mutant β-cDNA and treated with lactacystin, pro β band is stronger, (M) COS cells transfected with pIRES2-EGFP carrying mutant β-cDNA, (W) COS cells transfected with pIRES2-EGFP carrying wild-type β-cDNA. The positions corresponding to the pro β (65 kDa, denoted by filled arrow head) and mature β (28 kDa, denoted by open arrowhead) chains are indicated on the right.

(ER form) and the processed mature β-chain (lysosomal form), whereas the Δ12 bp mutant-tranfected cells produced weak band related to either form of the β-chain. On the other hand, kifunensine and lactacystin treated extracts showed significant levels of the pro-β form of the subunit (Figure 3). Kifunensine and lactacystin inhibited ERAD of the mutant pro-β, but did not result in any enhancement of residual Hex activity (Table 1). Since there was no apparent increase in mature β-chains levels, it must be concluded that the increased levels of mutant protein remained unable to fold and be recognized by the ERA-transport machinery, which

Table 1. Hex activities in transfected COS-1 cells

	Hex activity nmol/h mg protein (% of wild type)	Western Blot Results
Wild β-cDNA transfected cells	106.78 (100)	Pro β (+++) Mature β (+++)
Mock transfected cells	37.04 (35)	Pro β (-) Mature β (-)
Nontreated, mutant β-cDNA transfected cells	40.08 (37)	Pro β (+) Mature β (-)
Kifunensine treated mutant β-cDNA transfected cells	41.56 (39)	Pro β (+++) Mature β (-)
Lactacystin treated mutant β-cDNA transfected cells	40.15 (37)	Pro β (++) Mature β (-)

(+) indicates intensity of the bands in Western Blot analysis, (-) determines no visible protein bands.

would have resulted in lysosomal compartmentalization and maturation.

CHO cells stably expressing the βD208N were treated with DMSO (Mock) or Kifunensine, pulse-labelled with ^{35}S-Met for 30 minutes and then chased (grown in media containing "cold" methionine) for periods of 2, 3.5 and 10 hrs (Figure 4). After a chase period of 10 hrs mutant β-precursor could be detected in cells treated with kifunensine at levels similar to those at the start of the chase period, whereas it was below detection limits in mock treated cells. Thus in the presence of kifunensine the half-life was much greater that 10 hrs, whereas in mock treated cells the half-life of the precursor was decreased to between 3.5 and 10 hrs. To examine the role of the proteasome on the half-life of the precursor, stably expressing cells were treated with Lactacystin or DMSO. Following a similar labelling approach, mutant β-precusor was readily observed in lactacystin-treated cells but not in DMSO-treated cells after an extended chase period of nine hours.

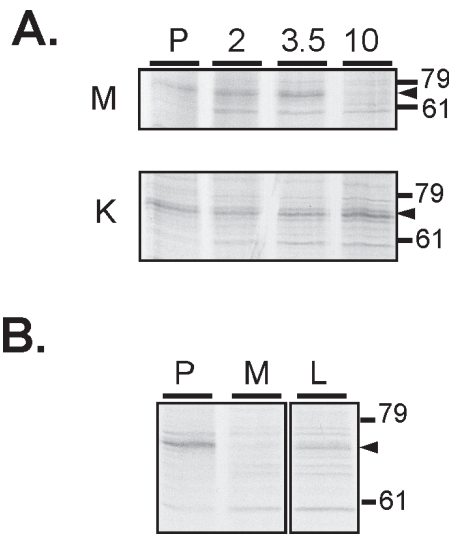

Figure 4. Half-life of D208N mutant Hex β-protein stably expressed in CHO cells in the absence or presence of kifunensine or lactacystin. CHO cells expressing the mutant protein were pre-treated with kifunensine or lactacystin during pulse labelling and chase periods. Radiolabelled (^{35}S-Met and ^{35}S-Cys) mutant protein was immunoprecipitated using rabbit anti-human Hex A bound to protein-G beads, followed by SDS-polyacrylamide gel electrophoresis and visualization of the corresponding bands by radioautography. A. Immunoprecipitated protein from Mock (M) or Kifunensine (K) treated cells following pulse labelling with ^{35}S-Met (P), or after chase periods of 2, 3.5 or 10 hours. B. Immunopreciptated protein from D208N Hex β-expressing cells immediately after pulse-labelling (P) and following a chase period of 9 hours in the absence (M) or presence of lactacystin (L). Position of molecular weight markers (in kilodaltons) and Pro- β band (denoted by filled arrow heads) are shown to the right of the panels.

Discussion

Two mutations, a deletion mutation Δ12bp (residues 398-401) and a missense mutation D208N were used to examine the terminal steps in the folding pathway of the Hex β-subunit. Deletion of residues INKG found in the linker between the alpha-helix (α4) and beta strand (β5) that are part of the α–β TIM barrel that defines the catalytic domain of Hex, has been shown to affect the folding of the β-subunit. The analogous Δ12 bp mutation in the pro-α-chain (ΔYGKG, residues 366-369) that has been identified in two Turkish Tay-Sachs patients, also results in misfolded protein that is retained in the ER. The mutant α-precusor chain does not undergo further proteolytic processing in the lysosome to its mature form (18). In the case of the D208N mutant, it is not clear how the mutation results in a nonfunctional, misfolded protein. However, on the basis of the 3D structure of Hex B, it is known that D208, interacts with and stabilizes Arg211, which is involved in substrate binding and catalysis. A mutation in the analogous Arg at position 178 in the α- subunit results in the B1 variant of Tay-Sachs disease.

When expressed in the aligned position in the β-subunit, the Δ12 bp mutation prevents even the inherently more stable pro-β monomers (as compared to pro-α monomers)

from being able to fold, dimerize and exit the ER for transportation to the lysosome, where they would be processed into their mature forms (19). If the misfolded protein is unable to adopt a native conformation, then there are two remaining destinies for the protein; it can be recognized and once-again bound by general ER-chaperones, such as calnexin/calreticulin, for another attempt at folding, or it can be recognized by the retro translocation machinery and exported for degradation by the proteasome (ERAD). Mutants that are in a futile folding cycle are susceptible to the action of ER α-mannosidase I which removes a mannose residue from the Man9GlcNAc2-oligosacharide. This step is inhibited by kifunensine and allows the mutant protein the chance to re-enter the folding cycle. Mutants bearing the Man8 oligosaccharide are recognized by EDEM-1 which is involved in exporting the misfolded protein from the ER. During retro translocation it is tagged with ubiquitin prior to proteolytic degradation by the proteasome. This last step is inhibited by lactacystin and represents the point of no return for the mutant protein. To investigate the intracellular degradation of mutant β-subunit, the effects of lactacystin and kifunensine on the processing of mutant β-subunits were determined. COS-1 cells transiently or CHO cells permanently expressing the mutant β- subunits of Hex were used as the cellular model for determining the effect of these agents on either the Δ12 bp or the D208N missense mutation. After treatment with either chemica, a substantial increase in the levels of pro-β chains, but not mature β-chains or residual enzyme activity, was observed for both mutant proteins. These results showed that mutant β proteins are substrates for the calnexin/calreticulin pathway and are ultimately degraded by the proteasome. These data also demonstrate that, at least for these mutations, increasing the levels of mutant protein in the ER does not result in any detectable enhancement of transport to the lysosome or residual enzyme. In contrast to our finding with the two β-mutations, Bartoli et al. demonstrated that kifunensine or lactacystin treatment were able to pharmacologically rescue the R77C mutation of the protein encoded by the sarcoglycan gene (SGCA) from ER-retention. In this study, muscle from (SGCA)-null mice was transfected with an adeno-associated virus vector coding for the human mutant R77C protein (37). Noticeably, this treatment did not present with any obvious toxicity. Importantly, the mutated R77C protein was correctly targeted to the plasma membrane following treatment with kifunensine, demonstrating therapeutic efficiency. These different outcomes following kifunensine treatment may be explained by the variations in the severity of each mutation's effect on protein folding.

In conclusion, this study provided clear evidence that in tranfected COS-1 or CHO cells, recognition and degradation of misfolded mutant β-subunits of Hex (Δ12 bp and D208N) occur through the well characterized calnexin/calreticulin and the ubiquitin-proteasome pathways, respectively. Additionally with these specific mutations, increasing the levels of misfolded protein in the ER does not result in increased levels of transport to the lysosome or residual enzyme activity.

Acknowledgements

We thank G. Luckacs, M. Sharma and S. Dougaparsad for advice and helpful discussions regarding immunoprecipitation and mannosidase/proteasome inhibition studies.

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