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Mutations alter secretion of fukutin-related protein

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Abstract

Mutations in the fukutin-related protein (*FKRP*) gene cause limb-girdle muscular dystrophy type 2I (LGMD2I) as well as other severe muscle disorders, including Walker–Warburg syndrome, muscle–eye–brain disease, and congenital muscular dystrophy type 1C. The *FKRP* gene encodes a putative glycosyltransferase, but its precise localization and functions have yet to be determined. In the present study, we demonstrated that normal FKRP is secreted into culture medium and mutations alter the pattern of secretion in CHO cells. L276I mutation associated with mild disease phenotype was shown to reduce the level of secretion whereas P448L and C318Y mutations associated with severe disease phenotype almost abolished the secretion. However, a truncated FKRP mutant protein lacking the entire C terminal 185 amino acids due to the E310X nonsense mutation was able to secrete as efficiently as the normal FKRP. The N-terminal signal peptide sequence is apparently cleaved from the secreted FKRP proteins. Alteration of the secretion pathway by different mutations and spontaneous read-through of nonsense mutation may contribute to wide variations in phenotypes associated with FKRP-related diseases.

Introduction

Recently, mutations in several genes have been associated with a sub-set of muscular dystrophies that share a common secondary abnormality in O-linked glycosylation of α -dystroglycan (α -DG), collectively known as dystroglycanopathies [1,2]. These genes include *fukutin*, *fukutin-related protein (FKRP)*, *LARGE*, *POMGnT1*, *POMT1*, and *POMT2* encoding known or putative glycosyltransferases [3-8]. Among the genes of particular interest is *FKRP*. Mutations in the *FKRP* gene cause a broad spectrum of phenotypes ranging from limb-girdle muscular dystrophy type 2I (LGMD2I) with mild clinical symptoms [4] to more severe congenital muscular dystrophy type 1C (MDC1C) [5, 9]. In addition, affected patients frequently develop cardiomyopathy [10]. *FKRP* also plays an important role in other non-muscle tissues. For example, *FKRP* mutations were reported in patients diagnosed with muscle-eye-brain disease (MEB) and Walker-Warburg syndrome (WWS), both of which are associated with severe brain defects and mental retardation in addition to muscle involvement [11-12]. Mechanism(s) underlying the wide phenotypic variations is not understood. The most noticeable molecular pathology of *FKRP*-related diseases is the secondary defects in glycosylation of α -DG as revealed by the reduction or loss of immunoreactivity to the IH6 and VIA4 antibodies, which recognize the sugar moiety of the protein [1, 2]. This leads to the assumption that *FKRP* may function as a glycosyltransferase. This hypothesis is supported by sequence analysis showing that *FKRP* contains a consensus putative catalytic DxD motif commonly found in many glycosyltransferases in the Golgi apparatus [13-14]. However, there is no direct evidence indicating that *FKRP* is a glycosyltransferase.

Previous sequence analysis predicts that FKRP is a type II single-pass transmembrane protein which anchors to the membrane and targets to the Golgi apparatus [13]. It has been known that transmembrane domains of a protein share common structural features with signal peptides. Sequence analysis with a program for protein structure/function prediction (Phobius prediction; phobias.cbr.su.se/cgi-bin/predict.pl) suggests that the transmembrane domain of FKRP is a signal peptide with the likelihood of the protein to be secreted. In agreement with the prediction, fukutin, another putative glycosyltransferase and sharing high sequence homologue with FKRP, was shown to be secreted into culture media [3]. However, the functional significance of the secreted fukutin is not clear.

Modifications of glycosyltransferases, such as formation of dimers and proteolytic cleavage and secretion as a soluble form, are considered potentially important processes to regulate the activity of glycosyltransferases [15-17]. For example, β 1,6-*N*-acetylglucosaminyltransferase V (GnT-V) secreted from human colon carcinoma cells is directly involved in tumor angiogenesis in a glycosylation-independent manner [18], suggesting that secreted glycosyltransferase could have distinctive function. In this study, we investigated whether FKRP is secreted and the potential effect of individual mutations on the secretory pathway. We showed that both normal human and mouse FKRP were secreted into the culture medium efficiently in CHO cells and mutations affected the secretion. Interestingly, spontaneous read-through was clearly detected for the E310X nonsense mutant, resulting in low level expression of full length FKRP. Our results suggest that alteration of the secretory pathway by individual mutation may add

another layer of complexity to the variation of phenotypes associated with FKRP-related diseases although potential biological functions of the secreted FKRP protein remain to be explored.

Materials and Methods

Construction of expression plasmids

Full length coding sequence of the human FKRP was amplified by RT-PCR from the total RNA isolated from a normal skeletal muscle. The cDNA was placed at the 5' end of the eGFP coding sequence in the pEGFP-N3 vector (Clontech). The subsequent removal of the GFP tag from the construct generated an untagged human FKRP expression vector. The human L276I and P488L mutant constructs were created by PCR-directed mutagenesis as described previously [19]. Same approach was used for generating the E310X FKRP construct with the following pair of primers; hfkpr-310/MS1-F1202-1222, 5'-C TAC CTC TAC TAG GAG CGC T-3' and hfkpr-310/MR1-R1222-1202, 5'-A GCG CTC CTA GTA GAG GTA G-3' (mutated nucleotide is in bold and underlined). The kozak sequence upstream of the GFP was removed to reduce background GFP level for read-through study. The expression constructs of mouse normal FKRP, L276I, C318Y and P448L mutants were tagged with eYFP as reported previously (13).

Cell culture and transfection

CHO cells (2×10^5 /well of 6-well plates) were grown in RPMI-1640 medium supplemented with 10% FBS, and 4mM L-glutamine. Twenty four hours after plating, cells were transfected with 5 μ g of each construct using Lipofectamine 2000 as per

manufacturer's instructions. After 24 hours, the cells were then grown in 2ml of Optipro SFM, 12309 (Invitrogen, Carlsbad, CA) supplemented with 4mM L-glutamine for 48 hours in 5% CO₂. The culture media were collected and pre-cleared by centrifugation at 13,000rpm for 15 minutes. The supernatant was subsequently concentrated from the original volume (2ml) to a smaller volume (250µl) using concentrator VIVASPIN, 10,000MWCO, (VIVASCIENCE, Massachusetts) according to manufacturer's instructions. The cells were collected separately by scrapping off from the culture plate and referred to as cell pellet. At least three independent transfections were performed to confirm the results. Transfected cells were also selected with G418 at the concentration of 1.5mg/ml and maintained as cell lines with stable expression of FKRP. All culture media and transfection reagents were purchased from Invitrogen/Gibco Life Sciences.

Antibodies and immunohistochemistry.

FKRP-5643 (FKRP-STEM) rabbit polyclonal antibody against the stem region (amino-acid residues 29-130) of the mouse FKRP was used as described previously [14]. The anti-GM130 monoclonal antibody was purchased from BD Biosciences (San Jose, CA). Monoclonal antibody against β -actin (clone AC-74, A 5316) and antibody against GFP/YFP (clone JL-8) were obtained from Sigma and Clontech respectively.

Cells were fixed in 2% paraformaldehyde in PBS at 4°C for 15 minutes at 24 hours post-transfection with 1µg each of expression construct. After thorough rinsing with PBS, the cells were first blocked with 10% normal goat serum and 10% fetal bovine serum diluted in PBS for 30 minutes, and then incubated for 1 hour at room temperature with the primary antibodies, FKRP-STEM (1: 500 dilution), GM130 (1:500). All antibodies were diluted in PBS containing 10% fetal bovine serum. Cells were then

incubated with AlexaFluor 594-conjugated anti-mouse or anti-rabbit secondary antibodies (Gibco/Invitrogen). Immunofluorescence was visualized using an Olympus BX51/BX52 fluorescence microscope and images were captured using the Olympus DP70 Digital Camera System (OPELCO, Dulles, VA).

Western blot analysis

Transfected cells were harvested 48 hours post-transfection. Total protein of the cell lysate was extracted in 400 μ l Triton lysis buffer containing 1% Triton X-100, 50mM Tris PH 7.5, 150mM NaCl, 2mM DTT and protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Equal volume (7.5 μ l) of cell lysate (20 μ g protein) and concentrated culture supernatant (250 μ l from a total of 2ml culture medium) were resolved on a 4-15% linear gradient Tris-HCl SDS PAGE gel (Bio-Rad Hercules, CA). The membrane was probed with primary antibodies followed by horseradish peroxidase-conjugated IgG secondary antibodies (Bio-Rad). Western blots were performed according to the procedure described previously [19]. β -Actin (A5316, Sigma-Aldrich) was used as a control for protein loading. The signal intensity of the bands was measured by NIH ImageJ software. Total amount of FKRP in both culture supernatant and cell lysate of the same culture is considered as 100% and the % of FKRP in culture supernatant was adjusted by the ratio of total sample volume (250 μ l of culture supernatant : 400 μ l cell lysate).

PNGase F Deglycosylation

Twenty μg of total cell lysate and equal volume of cultured media were boiled for 10 minutes in 1x Glycoprotein Denaturing Buffer (NEB, Ipswich, MA) followed by incubation with 500 units of PNGase F at 37°C for 6 hours. Samples were analyzed by Western blots as described above.

Microsome preparation

Cells transfected with FKRP were washed with PBS and homogenized in 500 μl of pyrophosphate buffer, pH 7.1 (20mM sodium pyrophosphate, 20mM sodium phosphate monobasic, 1mM MgCl_2 , 0.5mM EDTA and 10% sucrose) with freshly prepared protease inhibitor cocktail (Roche, Germany). The homogenates were centrifuged at 2,000g for 10 minutes at 4°C . Cell pellets were re-homogenized in same volume of buffer. The supernatants from the two centrifugations were combined and subject to ultracentrifugation at 100,000g for 1h at 4°C . The final pellets were dissolved in TX-100 buffer (1% Triton-100, 50mM Tris, pH 8, 150mM NaCl and 0.1% SDS) and analyzed with the supernatant on SDS-PAGE gel. Culture medium from FKRP-transfected cells was also ultracentrifugated at 100,000g for 1h at 4°C . Final pellet was dissolved in TX-100 buffer and analyzed with the supernatant on SDS-PAGE gel.

Results

Normal FKRP is secreted into culture medium

FKRP contains a transmembrane domain and a conserved R/KxxR/K motif in the N-terminus (Fig. 1). Previous studies have shown that the arginine at amino acid position 2 (R2) and 5 (R5) immediately adjacent to the transmembrane domain are critical for Golgi localization [13]. Protein sequence and structure analysis predicts that the transmembrane domain is a signal peptide (Phobius prediction; phobias.cbr.su.se/cgi-bin/predict.pl) (Fig. 1). This suggests that FKRP might be secreted. To investigate this possibility, CHO cells were transfected with the normal human FKRP tagged with a GFP reporter which has been widely used as a tag for tracking the pathway and function of target proteins. Our previous study has confirmed that the GFP tag did not alter protein localization [13-14]. Equal volume of the cultured medium and the cell lysate were then analyzed in parallel by Western blot for the presence of FKRP. FKRP proteins were detected as both monomers (~75kDa) and dimers (~150kDa) in the cell lysate. FKRP with the expected size of monomers, but not the dimers were clearly detected in the culture medium (Fig. 2A). The signal intensity of the FKRP in the culture media was similar to that (both monomers and dimers) in the cell pellet as measured by the NIH ImageJ. The amount of FKRP protein in the culture medium was therefore approximately 40% of the total FKRP detected in both medium and cell pellets after taking the total volume of cell lysate (250 μ l) and culture medium (400 μ l) into account (Fig. 2C) (the same factor will be applied to calculate the % of secreted mutant FKRP hereafter). The identity of the FKRP-GFP fusion protein was also confirmed by antibody to GFP (Fig. 2D). FKRP were also secreted into the

medium in the cells stably expressing the transgene with similar percentage (Fig. 2E). Consistent with our previous report [19], immunohistochemistry showed that normal human FKRP was predominantly localized within the Golgi apparatus (Fig. 3).

Missense mutations reduce the levels of FKRP secretion

Previous studies have demonstrated that mutations in FKRP cause mislocalization of mutant FKRP to the ER compartment (14,19). To determine whether mutations also affect secretion, we examined the effects of two FKRP mutations, L276I and P448L, which are associated with mild and severe clinical phenotypes respectively. Under the same culture condition, the levels of mutant FKRP proteins from the cell pellet were similar to that in the cells expressing normal FKRP (Fig. 2A). In contrast, the signal intensity of the L276I in the culture media was only about 22% of that in the cell pellet. The amount of L276I mutant FKRP in the medium was therefore approximately 15% of total mutant protein. Most significantly, the P448L mutant FKRP was barely detectable in the culture medium with the proportion corresponding only 3% of total mutant protein (Fig. 2C). We also observed that GFP was independently translated, probably utilizing IRES sequence upstream of the GFP coding region (Fig. 2D). However, the levels of the GFP in the medium were comparable between the cells expressing normal FKRP and mutant FKRP (Fig. 2D). These results, therefore, indicate that secretion of FKRP is independent to the level of the expression and secretion of other proteins.

We then examined the secretion of mouse FKRP and the same two mutants in the CHO cells. Similar pattern of secretion was observed with the normal FKRP being abundant whereas the mutant P448L protein was barely detectable in the medium

despite high levels of expression in the cell pellet (Fig. 2F). We also examined the C318Y mutant, which is associated with severe disease phenotype in WWS (13). The mutant protein localized both at the Golgi and ER with diffuse cytoplasmic signals [13]. Only about 10% of the C318Y protein was detected in the medium compared to about 24% and 80% of the P448L mutant and normal mouse FKRP detected in the media respectively (Fig. 2G). Thus, secretion is not limited to the human FKRP and reduction in secretion may be correlated to disease severity.

FKRP with a nonsense mutation retains in the Golgi and is secreted

Stable CHO cell lines were created to express the E310X (G928T) FKRP/GFP. Despite the presence of the nonsense mutation, two truncated polypeptides with the size of approximately 40KDa and 35KDa were expressed. Interestingly, only the 35KDa E310X FKRP was secreted into the medium, with the proportion accumulated within 48 hours reaching levels similar to that detected in the medium of normal human FKRP (Fig. 2A, C). GFP was only weakly detected in the medium, partly due to the absence of the kozak sequence in this vector, probably resulting in decreased level of independent translation (Fig. 2D). The size difference between the two species of truncated FKRP was less than 5Kd. This raised the possibility that the difference was caused by variations in glycosylation with the smaller form lacking N-glycosylation. However, PNGaseF digestion showed that the molecular weight of both truncated FKRP in the cell pellet as well as the secreted form were reduced by approximately 5 Kd, indicating that both species were similarly N-glycosylated (Fig. 4A).

Interestingly, a weak band equivalent to the size of full length FKRP was also

detected in the cell pellets of E310X transfectant by the antibody to the Stem region of the FKRP (asterisk in Fig. 2A). The signal intensity corresponded to less than 5% of the total truncated FKRP detected in the cell pellet (Fig. 2C). This band was also detected by the antibody to GFP, indicating that the 75Kd band represents the full length FKRP/GFP fusion protein (Fig. 2D), probably by the read-through mechanism. However, full-length FKRP/GFP protein was not detected in the culture medium and this could be due to limited amount of the protein within the cells. Immunocytochemistry demonstrated that the E310X mutant localized to the Golgi in almost all transfected cells although weak diffused signals within cytoplasm were also present in some cells (Fig. 3). These results therefore further support that the presence of GFP tag did not affect FKRP secretion and that alteration in FKRP transportation was dependent on specific mutations of the protein.

N-terminal signal peptide is cleaved from the secreted FKRP

The size difference of about 5KDa between the two truncated E310X FKRP species raises the probability that the smaller species represents FKRP with the signal peptide sequence cleaved. This would be consistent with the Phobius prediction that the N-terminal peptide sequence of the FKRP is cleaved at the position between amino acid 27 and 28 (probability, 0.85). The difference in size between the cleaved and non-cleaved FKRP is estimated to be approximately 3KDa. Since SDS-PAGE gel might not be able to distinguish small difference in size between proteins with molecular weight higher than 70kDa (as shown in Fig. 2A), we transfected CHO cells with the untagged FKRP and performed the Western blots using similar amount of FKRP proteins from

both culture medium and cell pellet. The molecular weight of the wild-type FKRP without the GFP tag was ~50kDa and the size of the secreted FKRP became noticeably smaller than the cellular form in the pellet. The difference in size between the two species of FKRP became more apparent after PNGaseF digestion (Fig. 4B). Similar reduction in size was observed for both species after digestion with PNGaseF, indicating that both species contain N-glycosylation (Fig. 4B). Thus, the reduction in size of the FKRP is likely the result of cleavage of the signal peptide, not differential glycosylation.

We also analyzed the presence of FKRP in different fractions of cell pellet and culture medium after ultracentrifugation at 100,000g. Transmembrane proteins will be retained in the pellet because of the hydrophobicity of the membrane segments and thus, easily separated from non-transmembrane proteins, which are typically retained in the supernatant. As shown in Fig. 5, FKRP in culture medium was present only in the supernatant fraction (lane 2) after ultracentrifugation, indicating the absence of its N-terminal transmembrane domain. In contrast, cellular FKRP together with the Golgi marker, GM28, were found only in the pellet (Fig. 5, lane 3). The cellular FKRP was also slightly larger in size compared to the FKRP secreted in the culture medium. These biochemical features further support our conclusion that the transmembrane domain was cleaved from the secreted FKRP, but intact in the cellular FKRP.

Discussion

In the present study, we demonstrated that normal FKRP is secreted into culture medium and mutations can alter the pattern of transportation. The L276I mutation, often associated with mild phenotypes in LGMD2I, caused a moderate decrease in secretion whereas the P448L and C318Y mutations, associated with severe phenotypes, dramatically reduced the secretion of the mutant proteins. The underlying mechanism(s) for the altered pattern of secretion of the mutant FKRP is not clearly understood. Our previous studies demonstrated that normal FKRP was transported and targeted to the Golgi apparatus [13-14, 19]. The L276I mutant was also primarily targeted to the Golgi and less susceptible to proteasomal degradation. In contrast, the P448L mutant was shown to accumulate in the endoplasmic reticulum (ER) where it was preferentially degraded in the proteasome (14). It is therefore possible that ER retention of the mutant proteins reduces the amount of FKRP secretion. This is consistent with the fact that the E310X mutant retains in the ER and secretes efficiently. However, the levels and size of both L276I and P448L mutant proteins were similar to normal FKRP and no clear degradation products were demonstrated on Western blots. Furthermore, the C318Y mutant demonstrated even lower levels of secretion when compared to the P448L mutant despite the fact that the protein still partially localizes to the Golgi. Thus, the relationship between secretion and ER retention of the mutant proteins remains to be elucidated.

Detection of secreted FKRP raises the question whether the N-terminal hydrophobic domain is cleaved from the FKRP protein. FKRP has been previously described as a type II transmembrane protein with its N-terminal a transmembrane

domain (N-terminal outside and C-terminal inside of the ER lumen) (13). Our previous study showed that the N-terminal 33 amino acids of FKRP are sufficient to recruit a GFP chimera to the Golgi apparatus. Similar results were obtained with the N-terminus of fukutin fused to GFP [13]. The remaining luminal sequence of FKRP fused to GFP was unable to target the Golgi apparatus, resulting in diffused distribution within the cytoplasm. It is therefore suggested that the protein is targeted to the Golgi apparatus by a non-cleavable signal anchor sequence [13]. Our results now suggest that the N-terminal signal peptide can be liberated from the secreted FKRP. However, it remains to be determined how and when the peptide sequence is cleaved in the secretory pathway. The observation that normal and E310X FKRP both localize predominantly to the Golgi apparatus with the signal peptide attached makes it plausible that the cleavage may occur after the proteins reach the Golgi apparatus. Interestingly, a high proportion of E310X mutant FKRP in the cell pellets has the signal peptide cleaved whereas cleavage is not apparent for the normal and other two mutant FKRP in the cell pellets. This raises the possibility that different mutations could differentially affect the cleavage of the signal peptide [20-22].

Mutations in FKRP are associated with muscular dystrophies ranging from MEB, WWS and MDC1C, with early manifestation and fatal consequence, to the mild LGMD2I [4,5, 9-12]. Asymptomatic carriers have also been reported [23]. The mechanisms by which mutations in FKRP cause such wide variations in clinic manifestations are not clearly understood. Our previous results demonstrated that FKRP mutants (S221R, A455D, P448L) associated with more severe clinical phenotypes are generally retained within the ER whereas the normal FKRP and the L276I mutant are found predominantly

in the Golgi apparatus [13, 14]. It was therefore suggested that the degree of ER-retention and preferential degradation by the proteasome might explain the phenotypic variations among muscular dystrophy patients with different FKRP mutations [14]. However, Dolatshad *et al* reported that both normal and mutant human FKRP including those associated with MDC1C, can be localized to the Golgi. They suggested that other factors besides mis-localization may also play important roles in the pathogenesis of the FKRP mutations [24, 25]. For example, FKRP has also been reported in association with the dystrophin-glycoprotein complex at the sarcolemma, suggesting a novel function at the muscle membrane [26]. In this study, we demonstrated that the E310X mutant lacking the entire C-terminal 185 amino acids localized mainly at the Golgi apparatus and is secreted with comparable efficiency to the normal FKRP. Although the deleted C-terminal sequence contains the putative catalytic DXD motif, the E310X mutation together with a L276I mutation is clinically associated with mild LGMD2I (case unreported). These results together clearly demonstrate the complexity of the effects of FKRP mutations on the localization, secretion and function of the protein.

In summary, our results confirmed that FKRP is an N-glycosylated protein and demonstrated that FKRP is secreted with the signal sequence apparently cleaved. Mutations alter the pattern of secretion. Alteration of the secretion pathway and translation by individual mutation may also partly account for the wide variations in clinic phenotypes associated with FKRP-related diseases.

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Figure legends

Fig. 1. FKRP protein sequence. Asterisks indicate the R2 and R5 amino acids critical for Golgi localization. Signal peptide sequence predicted by Phobius prediction (phobias.cbr.su.se/cgi-bin/predict.pl) is underlined and franked by the bended arrows on top of the letters. N glycosylation sites (in box) are predicted by NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc/). The predicted cleavage site of the signal peptide is marked by the arrow between amino acids 27 and 28. (Signal IP 3.0, www.cbs.dtu.dk/services/SignalIP. Probability, 0.850). The DVD motif is bolded and underlined.

Fig. 2. FKRP secretion in CHO cells. S/N, supernatant from cell culture; C/P, cell pellets; nFKRP, normal human FKRP. A. Detection of FKRP with antibody FKRP-STEM. Parental CHO cells without vector transfection were used as control. B. β -actin was used as protein loading control. C. Percentage of FKRP in S/N and C/P. Total amount of FKRP in both S/N and C/P of the same culture is considered as 100%. D. Detection of human FKRP/GFP fusion proteins and GFP in S/N and C/P by monoclonal antibody JL-8 to GFP. E. Detection of normal human FKRP in S/N and in C/P from stably transfected CHO cells. F. Detection of mouse FKRP in CHO cells with antibody FKRP-STEM (upper panel). Normal, normal FKRP. β -actin was used as protein loading control (lower panel). G. Detection of normal mouse FKRP (mFKRP), C318Y and P448L mutants in CHO cells with antibody FKRP-STEM (upper panel). A duplicate of Western blot was probed with monoclonal antibody JL-8 to YFP (lower panel). YFP is predominantly present in the medium of all 3 transfectants. H. Percentage of FKRP in S/

N and C/P. Equal volume of 7.5 μ l sample was loaded from both S/N and C/P for all Western blots. Signal intensity of the bands in the Western blots was measured by NIH ImageJ. The % of FKRP has been adjusted by the total volumes of S/N and C/P as described in the Material and Methods. Asterisks in A and D indicate the bands corresponding to the full length FKRP/GFP expressed in the E310X transfected cells.

Fig. 3. Colocalization of FKRP expression and Golgi marker GM130 by immunohistochemistry. Top panel, control cells without transfection. Middle panel, cells transfected with normal FKRP expression vector (nFKRP). Bottom panel, cells transfected with E310X expression vector. Left column, cells were stained with antibody to GM130 and detected with Alexa 594-conjugated goat anti-mouse IgGs (red). Middle column, FKRP expression was detected by antibody FKRP-STEM (green). Right column, GM130 merged with FKRP. Blue, nuclear staining.

Fig. 4. Deglycosylation of FKRP with PNGase F digestion. S/N, supernatant from cell culture; C/P, cell pellets. A. Human E310X mutant FKRP detected with antibody FKRP-STEM (upper panel). β -actin is used as protein loading control (lower panel). -, without PNGase F digestion; +, after PNGaseF digestion. B. Untagged normal human FKRP detected with antibody FKRP-STEM (upper panel). β -actin was used as protein loading control (lower panel).

Fig. 5. Normal FKRP detection in microsome preparations. Fractions of culture medium (S/N) or cell lysate (C/P) were collected by ultracentrifugation (100,000g) and analyzed by Western blots using antibodies against FKRP (A). Actin (B) was used as a control for S/N; GM28 (C) and hsp70 (D) were used as controls for C/P. Pellet and supernatant (Sup) were collected from both culture medium and cell lysate after ultracentrifugation. Note that the lower band of the cellular FKRP in the pellet of cell might represent degradation product. The cytoplasmic protein, hsp70, was predominantly found in the supernatant fraction of the cell lysate.

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 MRLTRCQAALAAAITLNLLVLFYVSWLQHQPRNSRARGPRRASAAGPRVT
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ACCEPTED







