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Using a ubiquitin ligase as an unfolded protein sensor

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ABSTRACT

A significant fraction of all proteins are misfolded and must be degraded. The ubiquitin–proteasome pathway provides an essential protein quality control function necessary for normal cellular homeostasis. Substrate specificity is mediated by proteins called ubiquitin ligases. In the endoplasmic reticulum (ER) a specialized pathway, the endoplasmic reticulum associated degradation (ERAD) pathway provides means to eliminate misfolded proteins from the ER. One marker used by the ER to identify misfolded glycoproteins is the presence of a high-mannose (Man5-8GlcNAc2) glycan. Recently, FBXO2 was shown to bind high mannose glycans and participate in ERAD. Using glycan arrays, immobilized glycoprotein pulldowns, and glycan competition assays we demonstrate that FBXO2 preferentially binds unfolded glycoproteins. Using recombinant, bacterially expressed GST-FBXO2 as an unfolded protein sensor we demonstrate it can be used to monitor increases in misfolded glycoproteins after physiological or pharmaceutical stressors.

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1. Introduction

The maintenance of the cellular proteasome is vital for cellular homeostasis. Protein production represents the final step in the flow of genetic information; hence, proteins reflect the sum of errors not only in transcription, splicing, and translation, but also in protein folding, which occurs in the endoplasmic reticulum (ER). High levels of misfolded proteins can disrupt cellular homeostasis, stress the ER, and dysregulate apoptosis. Indeed, the accumulation of specific misfolded proteins contributes to the pathologic sequela of many diseases, such as Alzheimer's, Parkinson's and Huntington's disease [1–5]. To date, assessing misfolding has only been possible at the level of individual proteins, and a variety of methods have been tried including native and non-native antibodies, circular dichroism, and fluorescence and infrared spectroscopy. It has not been possible, however, to measure the overall burden of protein misfolding placed upon a cell in a normal vs. a stressful situation.

One means to measures the levels of misfolded proteins within a cell may be to examine their attached sugars. Glycoprotein folding occurs in the ER with the assistance of the co-translational addition of oligosaccharides. This 14 sugar oligosaccharide, Glc3Man9(GlcNAc)2, is transferred en bloc to an asparagine residue on a nascent polypeptide. As these glycosylated proteins assume their tertiary structure, the inner GlcNAcGlcNAc core, also called chitobiose, of the attached glycans is sequestered, leaving only terminal sugars exposed [6–8]. Correctly folded glycoproteins are exported to the Golgi for further processing; incorrectly folded proteins are retro-translocated and degraded by the ubiquitin proteasome pathway. The cellular signal for retro-translocation is, in part, the exposed glycan core [9]. We thought perhaps that using the glycan attachment as a readout might provide a means for measuring overall levels of misfolded glycoproteins.

We wanted to test if the exposed core sugar groups can be exploited to quantify the levels of misfolded glycoproteins. Exposed glycan cores can be recognized by the lectin like ubiquitin ligase FBXO2. FBXO2 was first shown by Yoshida et al. [10] to bind glycoproteins. Subsequently, this group [11–13] and ours [14,15] showed FBXO2 preferentially binds glycoproteins through their glycan moiety and not by recognizing the protein component. We thought perhaps if we could prove that FBXO2 bound only unfolded glycoproteins, and that it bound a large array of unfolded glycoproteins, we could use FBXO2's ability to bind unfolded protein to determine the levels of unfolded proteins within a cell.

2. Materials and methods

All supplies are from Sigma–Aldrich (St. Louis, Mo.) unless otherwise indicated.

2.1. Cell culture

COS-7(American Type Tissue Collection (ATTC), Rockville, MD) cells were maintained in DMEM with 10% fetal bovine serum (FBS) (Gibco-BRL, Carlsbad, CA). Cell lines were incubated at

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37 °C, in 5% CO_2. As peviously described [16]. Cells were treated with 100 μM kifunensin, 10 mM 3-Methyl-Adenine, 10 nM epoxomycin (Boston Biochem), 10 μM lactacystin(Boston Biochem), 10 μM MG-132, 3.6 μM bafilomycin A1 for 18 h then harvested as below.

2.2. Cell lysates

Cells were rinsed with ice-cold PBS and incubated on ice with Flag Lysis Buffer (FLB: 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 containing EDTA-free protease inhibitors (Complete, Roche, Alameda, CA) for 10 min. Cells were then scraped off the plate and incubated on ice for 30 min, vortexing every 5 min. Lysates were cleared by centrifugation at 16,000*g* for 15 min.

2.3. Sugar competition assay

Lysates of COS-7 cells expressing FLAG-FBXO2 were processed as described above. Lysates were passed over beads coated with anti-FLAG antibody and incubated at room temperature for 30 min, with end over end rotation. The beads were then transferred to a Handee Micro-Spin column (Pierce, Rockford, IL), washed 4× with 200 μL of FLB. Then the indicated amounts of mannose, GlcNAcGlcNAc or Man3GlcNAcGlcNAc (V-Labs, Covington LA) or FLAG peptide eluted the complexes. As a control for GNA binding, aliquots of the FLAG or Man3GlcNAcGlcNAc eluates were heat denatured 10 min at 95 °C in 0.5× SDS-DTT denaturing buffer and then incubated with PNGaseF (NEB) for 1 h. Eluates were separated by SDS–PAGE, transferred to PVDF membranes and analyzed by GNA, as described below.

2.4. GST-fusion purification

Recombinant GST-FBXO2 proteins were purified from OverExpress C41 cells (an *E. coli* strain selected for tolerance to toxic proteins; Lucigen, Middleton, WI). Cells were grown overnight at 37 °C in MagicMedia (Invitrogen, Carlsbad, CA), and then for 6 h at 30 °C. Cells were pelleted and lysed for 30 min in 30 ml BugBuster (Novagen, Gibbstown, NJ)/500 ml cell culture, with protease inhibitors (Complete), as per the manufacturers protocol. After centrifuging at 16,000*g* for 20 min at 4C, the supernatant was collected. PBS equilibrated Glutathione Sepharose 4B beads (1.0 ml slurry/500 ml cell culture) (Amersham, Piscataway, N.J.) were added and the lysates rocked for 30 min at room temperature. The beads were pelleted at 500*g* for 5 min and washed with 10 bed volumes of PBS. Purified GST fusion proteins were eluted with 700 μL of reduced glutathione (10 mM) for 10 min at room temperature. The GST-fusion proteins were concentrated by size filtration using a Microcon YM-100 filter (Millipore, Bedford, Ma.). Protein concentration was determined at OD 280 nm, using absorbance of 1 = 0.5 mg/ml. 600 μg of purified GST-FBO2 protein was shipped on dry ice for analysis by the glycan array. Purified protein was stored at −80 °C until used.

2.5. Glycan array

1.0 l of GST-FBXO2 cell culture was used for analysis by the Consortium for Functional Glycomics sponsored-glycan array. Details of glycan array procedures and full data sets are available at the Consortium for Functional Glycomics web site (http://www.functionalglycomics.org/fg/). Purified GST-FBXO2 was added to the arrays at 30 μg/ml. Binding was detected by incubating with 25 μL goat anti-GST-FITC at 5 μg/mL. GST-FBXO2 was analyzed with plate EA v3.5. Image intensities were detected with a ScanArray confocal scanner.

2.6. Pull-down assay

Ten micro grams of GST-FBXO2 fusion protein was applied to 40 μg of kidney lysate. The samples were rotated end-over-end overnight at 4 °C. Fifteen micro lites of glutathione Sepharose beads was added for 30 min at room temp. The beads were then transferred to a Handee Micro-Spin column, washed 4× with 200 μL of FLB and eluted with 40 μL Laemmli buffer at 65 °C for 15 min.

2.7. Tissue lysates

Mice were anesthetized with Xylocaine/Ketamine, and perfused with cold PBS plus protease inhibitors. The organs were quickly removed, placed on dry ice, dissociated with a Polytron homogenizer, on ice in 2 μL of FLB/mg of dry weight with protease inhibitors. Debris pellets were removed after centrifugation at 16,000*g*, 4 °C for 15 min. Samples were stored at −80°C. For use in heat denaturation assays, 40 μg of tissue was heated to 60, 50, 40, or 25 °C for 15 min, cooled on ice, and then used in the pull-down assay, as described above.

2.8. GNA analysis

Samples were resolved by SDS–PAGE and transferred to PVDF membranes. Samples were blocked in PBS with 2% TWEEN 20 for 2 min at room temperature. They were rinsed twice with PBS and then incubated with 6 μg of snowdrop lectin (GNA; EY Laboratories, San Mateo, CA) in 0.05% TWEEN 20 with 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 1 mM $MgCl_2$, for 1 h at room temperature, and developed by chemiluminescence (Western Lightning; Perkin Elmer, Waltham, MA).

3. Results and discussion

3.1. Results of sugar competition assay demonstrate a preference for GN2-MAN3

To characterize the specificity of the FBXO2 sensor we used the eukaryotic N-terminal FLAG-FBXO2 fusion protein we had previously constructed to test the ability of various sugars to competitively elute off natural substrates. We transfected FLAG-FBXO2 into COS-7 cells and after 48 h lysed the cells with RIPA buffer in the presence of protease inhibitors and incubated with anti-FLAG antibody coated agarose beads. The agarose bead were transferred to a filter column and we eluted off the FLAG-FBXO2 bound substrate with various sugar groups, or FLAG peptide itself, and then ran the elutants on an SDS–PAGE gel and probed with GNA [7], a lectin that recognizes high mannose glycans (Supplemental Fig. 1, and Fig. 1A). As a control for GNA binding specificity an aliquot of FLAG and chitobiose-mannose core, (GN2Man3) eluants were digested with PNGaseF, an glycosidase that cleaves between the first GlcNAc and the asparagine amino acid (Supplemental Fig. 2). Treatment with PNGaseF removed all GNA binding substrates indicating that the GNA bound bands seen in Supplemental Fig. 1, and Fig. 1A were indeed glycoproteins. We used ImageJ to measure the band intensities of the eluted sugars, set the amount eluted by FLAG peptide as 100%, and plotted these values on a bar graph (Fig. 1B). As shown in Fig. 1B, mannose even at 100 mM (Nab 100) only removed 14% of the FBXO2 bound substrates. The chitobiose core alone (GN2) was 50 times more effective at removing FBXO2 substrates; removing 5 times more substrate at 10-fold less molar concentrations of chitobiose (10 mM). However, the most effective at removing FBXO2 bound substrates was the chitobiose-mannose core, (GN2Man3), at 1.0 mM, it removed even more substrate than the FLAG positive control. At 0.1 mM the

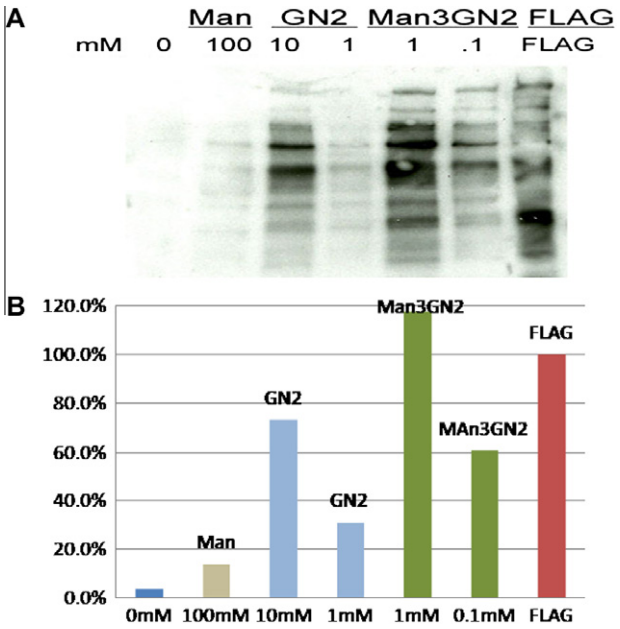

Fig. 1. FBXO2 bound glycoproteins are most effectively eluted with Man3GN2. (A) COS-7 cells were transfected with FLAG-FBXO2 harvested 48 h later, and FLAG-FBXO2 substrates were bound to anti-FLAG coated beads and substrates eluted off with indicated concentrations of sugars, and probed with the lectin GNA. (B) Image J analysis of SDS–PAGE gel FLAG elution set at 100%.

chitobiose-mannose core removed over 4 times more substrate than 100 mM mannose, thus it was 4000 times more effective than mannose alone, providing good evidence that FBXO2 recognizes and binds to the exposed chitobiose-mannose core. The multiple bands eluted on these SDS–PAGE gels indicate that FBXO2 is capable of binding multiple different proteins, and since this is a 1-D SDS–PAGE gel each band on the gel may represent multiple proteins, increasing the potential diversity of FBXO2 bound substrates.

3.2. Glycan array demonstrates FBXO2's exclusive affinity for chitobiose-mannose core

To use FBX02 as a glycoprotein sensor we added a GST moiety to the end-terminal region, distant from the glycan binding C-terminal portion of FBXO2. We then used this bacterially over-expressed and purified GST-FBX02 to see if the GST component interfered with glycan binding, by using a glycan array. Probing the glycan array with recombinant GST-FBXO2, we show the GST moiety does not affect the specificity of FBXO2 for core glycans (Fig. 2, Supplemental Fig. 3 and Table 1). Using the glycan array, we tested approximately 200 glycans and found an even more dramatic specificity than we had with our sugar elution discussed above. GST-FBXO2 had almost no affinity for either the mannose groups alone or for the chitobiose core alone and bound only those glycan containing a $GlcNAc_2$ core with 5–9 attached mannose groups. This is the core oligosaccharide exposed on unfolded glycoproteins [17–23]. This data is also consistent with our premise that by recognizing the chitobiose-mannose core FBXO2 may recognize a wide array of misfolded glycoproteins.

3.3. FBXO2 binds denature proteins

To assess whether FBXO2 can detect a broad range of misfolded glycoproteins, mouse kidney lysates ($n = 3$) were heated to partially denature the proteins and expose the core glycans. Bacterially over-expressed and purified GST-FBXO2 protein was mixed with heat denatured extracts, pulled down with glutathione beads and GST-FBXO2 substrates isolated, separated on SDS–PAGE and probed with GNA (Fig. 3A). Setting the amount of glycoproteins pulled down at room temp at 100% we found that heat denaturing to 40 °C resulted in a 86% increase in glycoproteins pulled down (Fig. 3B). Increasing the temperature to 60 °C increased the misfolded proteins pulled down to 237%, further increased in temperature actually resulted in less proteins pulled down as the denatured proteins began to precipitate out of solutions. The finding that heat denaturation increases the amount of proteins pulled down by FBXO2 suggests that FBXO2 preferentially recognizes misfolded proteins.

3.4. FBXO2 substrates increase under ER stress

While heat denaturation is similar in some aspects of protein misfolding, it is not entirely equivalent. To further demonstrate that FBXO2 binds misfolded glycoproteins we treated tissue cultures cells transfected with FLAG-FBXO2 with various chemical inhibitors of protein degradation and glycan processing known to

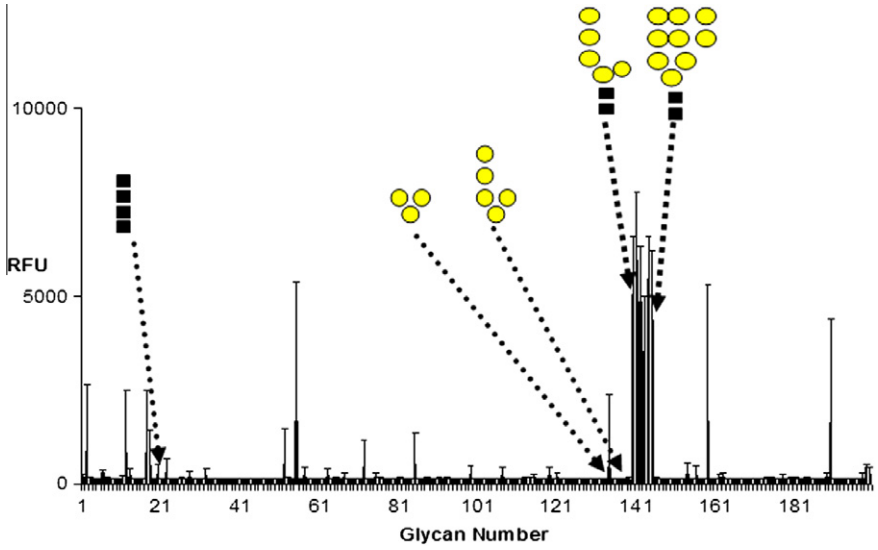

Fig. 2. Glycan array probed with GST-FBXO2 demonstrated only significant FBXO2 binding to chitobiose with attached mannoses. Bacterially expressed, purified GST-FBXO2 was added to a glycan array with 200 different sugars, washed and probed with anti-GST antibody. FBXO2, demonstrated significant binding only to glycans containing a GlcNAc core ■ (GN), with an attached mannose ● (Man), glycan numbers 138–145.

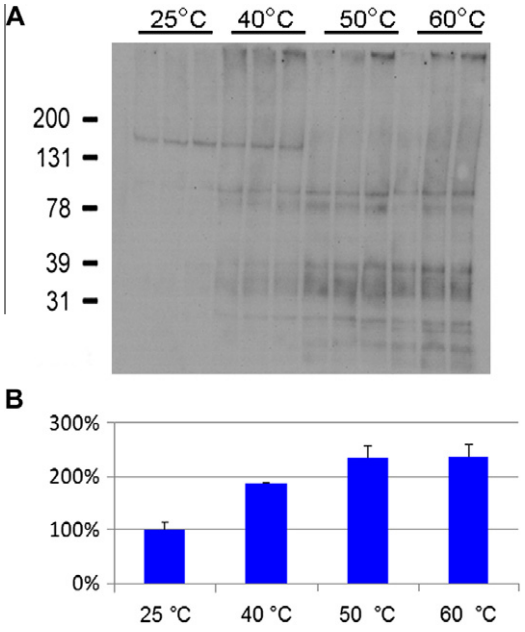

Fig. 3. Increasing denaturation temperature of a kidney lysate increases the levels of FBXO2 substrates. (A) Lysates from three different kidneys are shown, treated for the indicated temperatures and then incubated with GST-FBXO2. After elution off FBXO2, substrates were separated on SDS–PAGE gels and probed with GNA. (B) Image J analysis of SDS–PAGE gel 25 °C elution set at 100%.

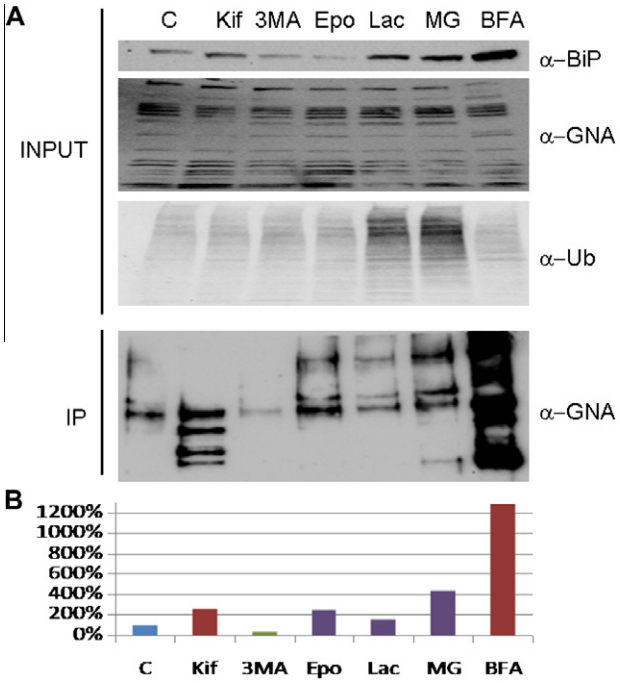

Fig. 4. Increasing ER stress increases FBXO2 substrates. (A) COS-7 cells were transfected with FLAG-FBXO2, treated with the indicated ER stressor, 100 μM kifeunensin (Kif), 10 mM 3-Methyl-Adenine (3MA), 10 nM epoxomycin (Epo), 10 μM lactacystin (Lac), 10 μM MG-132 (MG), 3.6 μM bafilomycin A1 (BFA), or vehicle (C) for 18 h then harvested. After anti-FLAG co-immunoprecipitation (IP) FBXO2 substrates were eluted and separated SDS–PAGE gels and probed with GNA. 10% of volume used in IP was run as total cell lysate (Input) and probed with indicated antibodies. (B) Image J analysis of SDS–PAGE gel control elution set at 100%, values normalized to GNA input.

result in protein misfolding, and performed anti-FLAG immunoprecipitation to detected FBXO2 bound substrates. Glycoproteins exported from the ER for protein degradation are degraded by the proteasome in a process known as ER associated degradation. Hence it was not surprising to find that the autophagy inhibitor, 3MA, did not result in an increase in misfolded glycoproteins (Fig. 4A).The mannosidase I inhibitor Kifunensine (Kif) resulted in an 260% increase in predominately lower molecular weight misfolded glycoproteins (Fig. 4B). The lactone antibiotic that inhibits ER to Golgi transport, Brefeldin A (BFA) resulted in the most dramatic increase in misfolded glycoproteins of 1300%. The three proteasome inhibitors, Epoxomicin (EPO), Lactacystin (LAC), and MG-132 (MG) resulted in an increase of 250%, 160%, and 440% as expected. Interestingly the increase in ubiquitin (Ub) staining which indicates successful proteasome inhibition did not parallel the FLAG-FBXO2 immunoprecipitation suggesting partial proteasome inhibition is sufficient to increase the levels of misfolded glycoproteins. Levels of the marker for ER stress BiP seemed to parallel levels of misfolded glycoproteins pulled down by FLAG-FBXO2, consistent with a model of ER stress leading to an accumulation of misfolded glycoproteins recognized by FBXO2. Input levels of GNA binding substrates, a marker for ER production were decreased in all experimental conditions. There are three possible reasons for such an unexpected result. Some cell lines process glycoproteins differently than other cell lines [24–27]. Other cell lines demonstrate no global changes to glycoprotein processing inhibitors but do report an increase in high mannose misfolded proteins, perhaps COS-7 cell are such a cell line [28–30]. And lastly overall glycoprotein production may have been decreased due to a reduction in ER efficiency as a result of ER stress [31]; since total protein loading was constant this would result in an apparent decrease in high mannose containing proteins.

Under normal conditions, up to 30% of all proteins are rapidly degraded by the proteasome, probably due to protein misfolding [32]. Moreover, it is notable that the levels of misfolded proteins are thought to be elevated in many diseases. Previous methods to monitor overall misfolding have assayed the degradation machinery via proteasomal or lysosomal markers and then extrapolated the extent of protein misfolding; these methods were indirect. Our experiments demonstrate that using the lectin like ubiquitin ligase FBXO2, we have developed a novel assay to measure the overall level of unfolded proteins directly. Measuring these levels of misfolded proteins gives us a powerful tool to assess perturbations in protein degradation because of disease, cellular stressors, or pharmacological manipulation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.109.

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