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A prominent role of PDIA6 in processing of misfolded proinsulin

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ABSTRACT

Despite its critical role in maintaining glucose homeostasis, surprisingly little is known about proinsulin folding in the endoplasmic reticulum. In this study we aimed to understand the chaperones involved in the maturation and degradation of proinsulin. We generated pancreatic beta cell lines expressing FLAG-tagged proinsulin. Several chaperones (including BiP, PDIA6, calnexin, calreticulin, GRP170, Erdj3 and ribophorin II) co-immunoprecipitated with proinsulin suggesting a role for these proteins in folding. To investigate the chaperones responsible for targeting misfolded proinsulin for degradation, we also created a beta cell line expressing FLAG-tagged proinsulin carrying the Akita mutation (Cys96Tyr). All chaperones found to be associated with wild type proinsulin also co-immunoprecipitated with Akita proinsulin. However, one additional protein, namely P58^{IPK}, specifically precipitated with Akita proinsulin and approximately ten fold more PDIA6, but not other PDI family members, was bound to Akita proinsulin. The latter suggests that PDIA6 may act as a key reductase and target misfolded proinsulin to the ER-degradation pathway. The preferential association of PDIA6 to Akita proinsulin was also confirmed in another beta cell line (βTC-6). Furthermore, for the first time, a physiologically relevant substrate for PDIA6 has been evidenced. Thus, this study has identified several chaperones/foldases that associated with wild type proinsulin and has also provided a comprehensive interactome for Akita misfolded proinsulin.

1. Introduction

The insulin molecule is synthesised as preproinsulin and translocated to the endoplasmic reticulum (ER), where immediately after translocation the signal peptide is cleaved by a signal peptidase to generate proinsulin [1]. Proinsulin contains three disulphide bonds; two disulphides linking the A and B chains of insulin and an additional intrachain disulphide bond within the A chain [2]. A number of ER-resident chaperones/folding enzymes, such as protein disulphide isomerase (PDI), are thought to assist in the folding of proinsulin to ensure the correct formation of disulphide bonds prior to its translocation from the ER to the Golgi apparatus [3,4]. In the trans-Golgi, proinsulin is assembled into proinsulin hexamers in the presence of zinc ions, and packaged into vesicles that bud from the trans-Golgi network [1,2]. Within the secretory granules proteolytic cleavage of the proinsulin hexamers by proconvertases and carboxypeptidases at the B chain/C-peptide junction and the A chain/C-peptide junction leads to the formation of insulin hexamers and C-peptides that are released into the circulation upon glucose stimulation [1,5].

In addition to its important role in controlling glucose homeostasis, insulin is a significant autoantigen in type 1 diabetes (T1D), however it is unclear what triggers the autoreactive immune response to proinsulin [6–8]. Aberrant forms of proinsulin may be more immunogenic than the native hormone as it has been shown that CD4 + T cells isolated from T1D patients specifically recognize misfolded proinsulin [9,10]. In addition, transgenic mice expressing mutant proinsulin (Akita mutation) where the cysteine at position 7 in the A chain, which normally engages in a crucial disulphide bond, is mutated to tyrosine develop diabetes [11,12]. In addition to this mutant form of proinsulin, Akita mice express wild type proinsulin from three alleles (two Ins1 and

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one Ins2), which should be more than sufficient to control glucose homeostasis and avoid diabetes. However, these mice experience beta cell loss a few weeks after birth and subsequently develop diabetes in the absence of inflammation [12,13].

Proteomics studies have shown that proteins thought to play a role in insulin biosynthesis (e.g. PDI, ERO1L-β) are altered in beta cells when exposed to proinflammatory cytokines [14,15]. We hypothesised that changes in the abundance of these molecules may lead to accumulation of misfolded proinsulin that in turn leads to ER stress. Very little is known about chaperone-assisted proinsulin folding and misfolding. A recent study by Pottekat et al. characterised 230 proteins that interacted with proinsulin/insulin during its biosynthesis [16]. Of these 230 proteins, 21 were found to interact with proinsulin at early stages of synthesis and folding [16]. However, an interactome for misfolded proinsulin has yet to be determined. Of note, Hartley et al. [17] identified genes that were affected by the inducible expression of mutant proinsulin in a beta cell line, but did not focus on investigating the proteins that interact with mutant proinsulin.

Here we were interested in identifying the foldases and chaperones involved in proinsulin production (folding and misfolding). FLAG-tagged proinsulin and mutant Akita proinsulin were expressed in the NIT-1 pancreatic beta cell line. Importantly, the tags provided unique immunoreactivity with anti-FLAG-monoclonal antibodies, and so enabled specific immunoprecipitation of proinsulin and associated molecules, irrespective of the conformation or processing of the introduced insulin molecule. Moreover, FLAG-tagged Akita proinsulin was specifically immunoprecipitated using this approach avoiding precipitation of endogenous wild type insulin associated with the use of insulin-specific antibodies. We identified several chaperones and foldases that associated with both wild type and Akita proinsulin and also a few that specifically associated with the mutant proinsulin. We also found evidence of a stable association between protein disulphide isomerase A6 (PDIA6), a PDI family member, and proinsulin which has not been directly reported in the past.

2. Experimental methods

2.1. Tissue culture

The SV40 transformed NIT-1 and βTC-6 insulinoma cell lines [18,19] were grown in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat inactivated foetal calf serum (FCS) (Sigma-Aldrich, St Louis, MO, USA), 2 mM L-glutamine (MP Biomedicals, Santa Ana, CA, USA), 100 units/ml benzyl-penicillin (CSL, Melbourne, Victoria, Australia), 0.1 mg/ml streptomycin sulphate (Sigma-Aldrich), 0.05 mM β-mercaptoethanol (Sigma-Aldrich), 5 mM HEPES buffer (MP Biomedicals) and 0.1 mM non-essential amino acids (Life Technologies) herein referred to as DM-10 at 37 °C and 10% CO_2. Transfected NIT-1 and βTC-6 cell lines were cultured in DM-10 supplemented with geneticin (0.5 mg/ml) (Life Technologies) to maintain the expression of the transfected gene.

2.2. Cloning and expression of FLAG-tagged proinsulin in NIT-1

Murine preproinsulin tagged with FLAG-at either C or N terminus was cloned into the *pcDNA3.1*(−) vector. In brief, proinsulin was PCR amplified using primers containing the FLAG-tag. Primers used are listed in Table 1. The amplified PCR products were ligated into *pcDNA3.1*(−) vector and the integrity of the sequences was verified by nucleotide sequencing. These constructs were used to transfect NIT-1 cells using Lipofectamine 2000 (Life Technologies). Selected clones were matched for expression of the transfected protein by western blot analysis.

2.3. Site directed mutagenesis, Akita proinsulin

Constructs encoding FLAG-tagged Akita mutant proinsulin, in which cysteine at position 96 in the A chain was mutated to tyrosine, were generated using the Quik change site-Directed Mutagenesis kit (Agilent Stratagene, Santa Clara, California, USA). In brief, 125 ng forward and reverse primer was added to PCR together with Pfu Turbo DNA polymerase and double stranded wild type FLAG-tagged proinsulin constructs as the template. Following PCR, *DpnI* was added to the tubes and transformed into XL1-Blue cells. The ampicillin resistant colonies containing the mutagenised construct were selected and verified by nucleotide sequencing.

2.4. Cloning and expression of FLAG-tagged wild type and Akita proinsulin in βTC-6

Flag tagged murine preproinsulin and Akita preproinsulin were cloned into the PIRES2Zsgreen1 vector. Briefly, the plasmids *pcDNA3.1*(−) containing the FLAG-tagged wild type and Akita proinsulin were digested with NheI and EcoRI to release the inserts and were then ligated into PIRES2Zsgreen1 vector. These constructs were used to transfect βTC-6 cells using Lipofectamine 2000. The transfectants were sorted to select for GFP positive cells and western blot analysis was performed to verify the expression of the transfected protein.

2.5. Immunoblotting

Cells were lysed at a density of 4×10^7 cells/ml in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Triton X-100 (LabChem, Zelienople, PA, USA) and complete protease inhibitor cocktail (Roche Applied Science, Castle Hill, NSW, Australia). Supernatants containing material from 2×10^5 cells were separated on 12% SDS-PAGE gels (Life Technologies) and the proteins transferred onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) in the presence of 20 mM Tris, 0.15 M glycine and 20% methanol. Membranes were blocked in 5% skim milk in phosphate buffered saline with 0.05% Tween (PBST) washed in PBST prior to probing with primary and secondary antibodies. Primary antibodies: anti-FLAG-(Sigma-Aldrich) 1:1000, anti-β Actin (Abcam, Cambridge, United Kingdom) 1:4000, anti-PDIA6 (Abcam) 1:1000, anti-PDI (produced in-house [20]) 1:10,000, and anti-calnexin (produced in house) 1:500. Proteins were visualised using Western Lighting Chemiluminescence (Perkin Elmer, Waltham, MA, USA) using LAS-3000 imaging system (FUJI, Japan). The bands on the membrane were quantitated using Image Quant (GE healthcare).

2.6. Fluorescent microscopy

Cells were plated onto sterile poly-L-lysine coated cover-slips at a density which would achieve a final confluence of 40–50% after

Table 1
List of primers.

FLAG-C-terminal	
Forward	5′CGCGCTAGCCCAGCCTATCTTCCAGGTTA
Reverse	5′GCCGAATTCCTACTTGTCATCGTCGTCCTTGTAGTCGTTGCAGT AGTTCTCCAGCT
FLAG-N-terminal	
Forward	5′CGCCTCGAGGACTACAAGGACGACGATGACAAGTTTGTCAAG CAGCACCTTTG
Reverse	5′GCCGAATTCGTGGGTCTAGTTGCAGTAG
Signal seq	
Forward	5′CGCGCTAGCCCAGCCTATCTTCCAGGTTA
Reverse	5′CGCCTCGAGAGCCTGGGTGGGGTGGGA
Akita mutation	
Forward	ATTGTAGATCAGTGC**TAC**ACCAGCATCTGCTCC
Reverse	GGAGCAGATGCTGGT**GTA**GCACTGATCTACAAT