Chapter 4

Selection and characterization of KDEL-specific VHH antibody fragments and their application in studying ER resident protein expression during ER stress

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Abstract

Several diseases are caused by defects in the protein secretory pathway, particularly in the endoplasmic reticulum (ER). These defects are manifested by the activation of the unfolded protein response (UPR) that involves the transcriptional up-regulation of several ER resident proteins, the down-regulation of protein translation and up-regulation of ER associated degradation (ERAD). Although this transcriptional up-regulation of ER resident proteins during ER stress has been described extensively, data on the differential protein expression levels of these same proteins are hardly available. Tools that would enable the simultaneous analysis of these proteins would be of high importance. Here, we describe the successful selection and characterization of VHH antibody fragments from a non-immune phage display library that recognize a conserved epitope present in several of these ER resident proteins, i.e. the C-terminal KDEL sequence, to study the differences in protein expression that occur during ER stress. In an ER stress model, involving treatment of HeLa cells with H_2O_2, DTT and tunicamycin, we show that the ER resident proteins endoplasmin and especially GRP78 are up-regulated on the protein level. In addition, our data show that there is a marked difference in the expression profile of KDEL-containing proteins after treatment with different stress inducers, which are probably related to the extent of ER stress.

Introduction

The ER is of critical importance for proper functioning of cells. Several diseases, such as familial hypercholesterolemia (FH) and cystic fibrosis (CF), are caused by problems that occur in the cellular secretory pathway (Hobbs et al., 1992; Kim and Arvan, 1998; Lukacs et al., 1994; Rutishauser and Spiess, 2002). Furthermore, there are indications that malfunctioning of the ER might even play a role in the aging process (Li and Holbrook, 2004; Rabek et al., 2003; van der Vlies et al., 2002). The protein secretion pathway involves several quality assurance and control mechanisms that ensure the correct folding and modification of secreted proteins, either membrane bound or soluble. The endoplasmic reticulum (ER) is a very important organelle in this process. Here, the newly translated proteins enter the protein secretion pathway where specialized proteins aid in the folding of the polypeptide chains into their correct conformation: Folding chaperones enable the correct folding of the amino acid chain, while other proteins are involved in the formation of disulfide bonds and other post translational modifications of the newly synthesized proteins. The importance of these processes is revealed during events that interfere with the correct folding or modifications of proteins in the ER (Schroder and Kaufman, 2005), as can be provoked by specific drugs (Pakula et al., 2003) and, as mentioned before, is reflected by the fact that several diseases are caused by malfunctioning of the ER (Kim and Arvan, 1998; Rutishauser and Spiess, 2002). When the protein folding capacity of the ER does not meet the demands, the ER responds by what is known as the unfolded protein response (UPR). The UPR consists of the activation of unique signal transduction routes via special sensors in the ER membrane in which the ER resident protein GRP78 plays an important role (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002). The UPR involves expansion of the ER, the transcriptional up-regulation of several ER resident proteins, the down-regulation of protein transcription and translation (Cox et al., 1997; Harding et al., 1999; Martinez and Chrispeels, 2003; Pakula et al., 2003), and up-regulation of ER associated degradation (ERAD) (Friedlander et al., 2000; Travers et al., 2000). When these processes cannot overcome the folding problems and the ER stress persists, the cells can eventually go into apoptosis (Orrenius et al., 2003; Yoneda et al., 2001).

The differential transcription of ER resident protein genes has been demonstrated in several diseases and in several model systems for ER stress

(Arvas et al., 2006; Kozutsumi et al., 1988; Martinez and Chrispeels, 2003). Although differential protein expression upon ER stress has been shown for some ER resident proteins (Hoozemans et al., 2005; Vattemi et al., 2004), it is less well described. To gain further insight in the processes that occur during ER stress, an antibody that has wide species specificity and that can recognize several ER resident proteins would be of high importance.

Many of the ER resident proteins contain special amino acid sequences that cause their specific retention in the ER. One of these ER-retention mechanisms involves the KDEL receptor, present in the *cis*-golgi that recognizes the amino acid sequence KDEL or a closely related tetra-peptide present at the C-terminus of several ER resident proteins (Munro and Pelham, 1987; Scheel and Pelham, 1996). Binding of a protein that contains this sequence to the KDEL receptor causes the uptake of the protein-receptor complex in COPI coated vesicles. COPI mediated retrograde transport of these vesicles to the ER and the subsequent release of the KDEL containing protein by the receptor, ensures the specific retention of the protein in the ER (Majoul et al., 1998; Wilson et al., 1993).

This ER retention signal enables the selection of antibodies with the before-mentioned characteristics, as this signal is conserved in several ER resident proteins and among many different organisms. In this study, we specifically selected and isolated single variable domain antibody fragments of heavy chain antibodies (VHH) from a large Llama-derived non-immune library that recognize the C-terminal amino acid sequence KDEL. The single domain structure of these antibody fragments simplifies the construction of a non-immune phage display library with wide antibody variability and enables high production yields in microorganisms (Frenken et al., 2000). A specific selection protocol was devised to drive the selection to the four amino acid KDEL epitope. To show the applicability of this antibody in the study of ER resident protein expression levels, KDEL containing proteins in HeLa cells were monitored during ER stress induced by H_2O_2, DTT or tunicamycin. The ER resident proteins endoplasmin and especially GRP78 show an up-regulation on the protein level in this ER stress model. Interestingly, there is a marked difference in the expression profile of KDEL-containing proteins after treatment with these different stressors, which is probably related to the extent of ER stress. The results illustrate that the obtained antibodies are a valuable tool in studying ER resident protein expression in ER stress models and ER-related

diseases. Furthermore, these results demonstrate the power of phage display in combination with single domain antibody fragments, as the obtained antibody fragments perform better than a commercially available anti-KDEL antibody obtained by hybridoma technology.

Materials and methods

Cloning and expression of recombinant proteins

The cDNA encoding Troponin C (TropC) was PCR amplified from a total human muscle cDNA preparation, with primers TropCforward 5'-CGGGATCCGATGACATCTACAAGGCTGCGG-3' and TropCreverse 5'-CCCAAGCTTCTCCACACCCTTCATGAACTCC-3'. For PCR, an initial denaturation step of 5 minutes at 95°C was followed by 25 cycles of 95°C for 1 minute, 62°C for 1 minute and 72°C for 2 minutes. The use of these primers introduced a 5'-*Bam*HI and a 3'-*Hin*dIII restriction site that allowed directed in-frame cloning in the pET28a expression vector (EMD Biosciences, Novagen Brand, Madison, U.S.A.). To introduce the amino acid sequence KDEL at the 3'-end of the Troponin C (TropC) gene, primers TropCKDELforward 5'-AGCTTAAAGATGAACTCTAAC-3' and TropCKDELreverse 5'-TCGAGTTAGAGTTCATCTTTA-3' were annealed and cloned behind the TropC gene with the restriction enzymes *Hin*dIII and *Xho*I. The obtained constructs were sequence verified (Sanger et al., 1977) and produced in *E. coli* strain BL21(DE3)-RIL (Stratagene, La Jolla, U.S.A.) according to standard procedures**.**

To construct a C-terminally KDEL-tagged version of glutathione S-transferase (GST), the pRP261 vector was used. This vector is a derivative of vector pGEX-3X (Amersham Biosciences, Roosendaal, The Netherlands). The primers GSTKDELforward 5'-GATCAAAGATGAGCTCTA-3' and GSTKDELreverse 5'-AGCTTAGAGCTCATCTTT-3' were annealed and cloned into pRP261 with the restriction enzymes *Bam*HI and *Hin*dIII. This cloning strategy resulted in a C-terminally KDEL-tagged GST in which the KDEL sequence was separated from the GST-encoding sequence by a factor Xa protease cleavage site. The expression construct was sequence verified and the KDEL-tagged GST was produced in *E. coli* strain DH5α according to standard protocols.

Purification of recombinant proteins

TropC with and without the C-terminal KDEL sequence was purified by means of its N-terminal His_6-tag using immobilized metal ion affinity chromatography (IMAC) according to the instructions of the manufacturer (Clontech laboratories, Mountain View, U.S.A.). The recombinant GST protein carrying the C-terminal protease factor Xa site and the KDEL sequence was purified with a VHH anti-GST column. VHH anti-GST was coupled to cyanogen bromide

(CNBr) activated sepharose 4B fastflow beads (Amersham Biosciences, Roosendaal, The Netherlands) according to the instructions of the manufacturer. Prior to use, the column was washed extensively four consecutive times with PBS of low (pH 2.0) and neutral pH (pH 7.4). Thereafter, a lysate of DH5α bacteria, induced to express the KDEL-tagged GST construct, was applied and the flow through was reloaded twice. Next, the column was washed twice with ten bed volumes of PBS pH 7.4. Bound protein was eluted by applying a total of three bed volumes PBS pH 2. The eluted fraction was neutralized with 1M Tris pH 7.5 and extensively dialyzed to PBS.

Selection of KDEL specific VHHs

Selection of VHHs specific for the C-terminal KDEL sequence was performed using a large Llama-derived (VHH) non-immune library, which was constructed from eight non-immunized Llamas and had a clonal diversity of about $5x10^9$. This library was kindly provided by Unilever Research Vlaardingen, The Netherlands.

To obtain KDEL specific VHHs, two consecutive rounds of phage panning were performed. For the first round, 1ug of GST-Xa-KDEL was coated overnight at 4°C in a well of a maxisorp plate (Nunc, Roskilde, Denmark). The following day, the well was washed three times with PBS containing 0.05% Tween20 (PBST) and blocked shaking at room temperature for one hour with 2.5% (w/v) Marvell (skimmed milk powder) in PBST.

Thereafter, the blocked well was incubated for two hours at room temperature with $3{\cdot}10^{12}$ colony forming units (CFU) of library phage, pre-incubated for 30 minutes in a solution containing 120μg/ml GST (Sigma-Aldrich, Steinheim, Germany) and 1.5% (w/v) Marvell in PBST. Wells were washed 20 times with 200μl PBST and two times with PBS. Phage bound to the KDEL sequence were specifically eluted by incubation with two units of factor Xa (Amersham Biosciences, Roosendaal, The Netherlands) in incubation buffer (50mM Tris/HCl pH 8.0; 150mM NaCl; 1mM $CaCl_2$) for one hour. Output phage were rescued essentially as described before.

For the second round of selection, 1μg of TropC-KDEL antigen was coated overnight at 4°C. The selection procedure was comparable to the first round of selection with the following adjustments; $1{\cdot}10^{10}$ CFU of rescued phage of the first selection round were used and no competition was performed during the incubation. After washing, bound phage were eluted with 100mM triethylamine

(TEA) for 10 minutes and the eluted fraction was neutralized with half a volume of 1M Tris/HCl pH 7.5. Output phage were used to infect exponentially growing *E. coli* TG1 cells and plated on LB agar plates containing 2% D-glucose and 100μg/ml ampicillin.

Primary evaluation of selected clones

Screening for KDEL-specific clones was performed by phage ELISA (Marks et al., 1991). Clonal phage were produced in 96 wells microtiter plates as described before and tested for their ability to bind 1μg GST-Xa-KDEL or TropC-KDEL coated on a maxisorp 96 wells plate (Nunc, Roskilde, Denmark). As negative controls, their KDEL lacking counterparts were used as antigen. Furthermore, a *Hin*FI DNA fingerprint was performed as described before (van Koningsbruggen et al., 2003). Antigen-reactive clones having a different *Hin*FI fingerprint pattern were sequenced (ServiceXs, Leiden, The Netherlands).

Re-cloning of anti-KDEL VHH

Monovalent VHH was obtained by transformation of the *E. coli* strain BL21(DE3)-RIL (Stratagene, La Jolla, U.S.A.) with the phagemid vector containing the VHH gene. This allowed expression of a monovalent VHH containing a C-terminal Myc- and His_6-tag, but without the bacteriophage gene3 protein fused to it.

Bivalent VHH were obtained by cloning the antibody fragment twice into the expression vector pUU-11 via two consecutive cloning steps. First, a *Pst*I restriction site was introduced in the framework 1 (FR1) region of the VHH-encoding genes by means of PCR with primers RK1 5'-GTGCAGCTGCAGGAGTCTGGGGGA-3' and MPE25WB 5'-TTTCTGTATGGGGTTTTGCTA-3'. The PCR product was cloned into the vector at the 3'-end of a triple alanine linker as a *Pst*I-*Bst*EII fragment. A second antibody fragment was cloned from the phagemid vector in front of this linker as a *Sfi*I-*Not*I fragment. The obtained construct was sequence verified. This allowed expression of a bivalent VHH with a triple alanine linker and a C-terminal Myc- and His_6-tag.

To obtain an antibody fragment that could specifically be immobilised on a solid support, VHH encoding genes were re-cloned into the expression vector pUR5850 (De Haard et al., 2005). This vector allows expression of C-terminal Myc and His_6-tagged protein in the periplasmic space of *E. coli* and it adds a

biotinylation sequence (LRSIFEAQKMEW) between these tags. The bivalent VHH was cloned into this vector as a *Sfi*I-*Bst*EII fragment via partial digestion from pUU-11. Upon expression of the construct in the *E. coli* strain AVB101 (Avidity, Denver, U.S.A.), which expresses the *BirA* gene under control of an IPTG-inducible promotor, the lysine in the biotinylation tag is biotinylated. Purification of the antibody fragments was carried out by means of their His_6-tag using IMAC as described above.

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen corporation, Breda, The Netherlands) containing 7.5% fetal calf serum, 100U/ml penicillin and 100μg/ml streptomycin, in a humidified atmosphere containing 5% CO_2 at 37°C.

Western blot analysis

For Western blot analysis, cells were grown in 10cm∅ dishes till 90% confluency, washed twice in PBS and lysed in 700μl lysis buffer [50mM Tris/HCl pH7.4; 100mM NaCl; 5mM EDTA; 1% (v/v) Triton X-100; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein quantification assay (Pierce, Rockford, U.S.A.). Equal amounts of protein were size-separated on a 10% poly-acrylamide gel and transferred to PVDF membrane. Before incubation with blockbuffer [2.5% (w/v) protifar plus (Nutricia, Zoetermeer, The Netherlands) in PBS], membranes were stained with ponceau red [0.1% (w/v) ponceau red; 0.5% (v/v) HAc] or coomassie brilliant blue (CBB) [0.1% (w/v) coomassie; 40% (v/v) MeOH; 10% (v/v) HAc] to confirm equal transfer of protein. Next, the membranes were incubated with the appropriate antibodies in block buffer. After every incubation with antibody, membranes were washed 5 times in PBST (0.05% Tween20 in PBS). After the last antibody incubation, membranes were washed two additional times with PBS. Bound antibodies were visualized by enhanced chemo luminescence (PerkinElmer, Boston, U.S.A.)

Immunoprecipitation (IP)

HeLa cells were grown till 90% confluency in 10cm∅ petri dishes, washed twice with PBS and lysed in 700μl IP buffer [10% (v/v) glycerol; 1% (v/v) NP40; 100mM NaCl; 50mM Tris/pH7.4; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein assay.

For IP with commercially available antibodies [αPDI (Benham et al., 2000), anti-KDEL (Stressgen, Victoria, Canada) or anti-Calreticulin (Stressgen, Victoria, Canada)], 500μg of HeLa cell lysate was incubated for two hours at 4°C with 3μg of antibody. Next, 20μl of a mix of proteinA and G beads were added to the cell lysate and incubated for an additional hour at 4°C.

For IP with the VHH antibody, 25μl streptavidin beads (Interchim, Montiuçon, France) were incubated for two hours with 5μg biotinylated bivalent VHH. Hereafter, beads were washed three times with twenty bed volumes of IP buffer and subsequently incubated with 1mg HeLa cell lysate for 4 hours at 4°C.

All beads were washed four times in twenty bed volumes of IP buffer. Finally, 50μl 2xSB [16% (v/v) glycerol; 0.15M DTT; 3.3% (v/v) SDS; 0.01% (w/v) bromphenol blue; 20mM Tris/HCl pH6.8] was added to the beads and beads were subsequently boiled for five minutes before Western blot analysis.

Protein identification

Immunoprecipitated proteins were loaded on a 12% in-house prepared SDS gel of 20 cm by using a Hoeffer gel system (Amersham biosciences, Roosendaal, The Netherlands). Proteins were visualized with Coomassie Brilliant Blue and bands of interest were sliced out of the gel and subjected to tryptic digestion (Shevchenko et al., 1996). Peptide extracts were identified with an Agilent 1100 LC system (Agilent, Palo Alto, USA) coupled to a Thermo Finnigan LTQ (Thermo electron Company, Waltham, MA, USA) as described previously in literature (Kolkman et al., 2005).

Immunofluorescence (IF)

HeLa cells were grown on 15mm∅ cover slips till 70% confluency. Cells were washed twice with PBS at 37°C and subsequently fixed with 4% formaldehyde in PBS for 20 minutes at room temperature. Cover slips were washed twice

with PBS for 5 minutes and cells were subsequently permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. The wash steps were repeated and non-reacted aldehyde groups were quenched with 50mM glycine in PBS for 10 min. Cells were washed twice with 1% BSA in PBS (blocking buffer) and subsequently blocked in the same buffer for 30 minutes. Thereafter, cover slips were incubated with the appropriate antibodies diluted in blocking buffer. The VHH anti-KDEL antibody was detected with a monoclonal anti-Myc antibody (9E10), followed by incubation with GAM-ALEXA555. The anti-PDI antibody was detected with GAR-ALEXA488. Each incubation with antibody was performed for one hour, subsequently followed by four wash steps with blocking buffer for 5 minutes. Finally, cells were washed twice with PBS, cover slips were mounted onto glass slides with Mowiol-PPD, air-dried and examined using a fluorescence microscope.

Induction of ER-stress

For stress resistance assessment, HeLa cells were grown in a 12 wells plate (Corning, U.S.A.) till 70% confluency and treated with decreasing amounts of dithio-threitol (DTT), hydrogen peroxide (H_2O_2) or tunicamycin (TM). The survival of the cells was followed in time. The highest concentration of the stress inducing agents where minimal cell death was observed was then chosen for further experiments. Cells were grown in 6cm∅ dishes till 70% confluency and treated for the indicated time points with DTT (1.5mM), H_2O_2 (50μM) or tunicamycin (10μg/ml). Treated cells were washed twice in PBS and lysed in 200μl lysis buffer [50mM Tris/HCl pH7.4; 100mM NaCl; 5mM EDTA; 1% (v/v) Triton X-100; protease inhibitors Complete (Roche Diagnostics, Mannheim, Germany)]. Non-soluble material was spun down (10.000g/ 5minutes/ 4°C) and the protein content of the supernatant was determined with a BCA protein assay (Pierce, Rockford, U.S.A.). Seven μg of each sample was analysed by Western blot as described above.

Results

Selection of KDEL-specific VHHs

Phage display was used to obtain VHH antibody fragments specific for the C-terminal amino acid sequence KDEL found in several mammalian ER resident proteins. Such antibody fragments could be used to monitor ER resident protein expression in biological samples. To this aim, a large Llama-derived (VHH) non-immune library was used with a clonal diversity of about $5x10^9$. The selection strategy used to obtain KDEL-specific VHH fragments, consisted of a combination of three steps during two selection rounds to specifically drive the selection towards this four amino acid epitope. (i) During the first round of selection, GST competition was performed during incubation of the phage library with immobilized GST-Xa-KDEL antigen. (ii) In addition, KDEL-bound phage were eluted by site-specific proteolysis with factor Xa, which cleaved off the C-terminal part (GIKDEL) of the coated protein. Both steps reduce the selection of GST specific antibody fragments and drive the selection towards enrichment of KDEL-specific VHHs. (iii) Furthermore, another KDEL-tagged antigen (TropC-KDEL) was used in the second round of selection. Only phage recognizing identical epitopes present on both antigens, i.e. the C-terminal KDEL sequence, should therefore be selected.

A

	Colony count 1st round	Colony count 2nd round
Input	$3•10^{12}$	$1•10^{10}$
Output KDEL	$2•10^{5}$	$6•10^{5}$
Output controle	$2•10^{4}$	$2•10^{4}$

B

	Output/input ratio	Signal/noise ratio
1st round	$6.6•10^{-8}$	10
2nd round	$6.0•10^{-5}$	30
Enrichment	909	

Table 1: Results of the selection against the C-terminal four amino acid epitope KDEL with a non-immune Llama phage display library. The amount of input and output phages of the first and second round are depicted (A) as well as the enrichment in output/input ratio and the increase in signal to noise ratio between the first and second round (B).

The titers of selected phage obtained in the first and second round of selection compared to background binding (signal/noise ratio), as well as the enrichment in output/input ratio, are depicted in table 1. Especially the latter indicated successful selection of KDEL-specific VHH fragments. Therefore, individual clones from this selection output were screened for antigen specificity. Phage of individual clones were tested in ELISA for binding to coated GST-Xa-KDEL and TropC-KDEL. About 50% of the tested clones gave

ELISA signals of at least four times the background on the KDEL-tagged antigens and signals comparable to background on the non-tagged proteins, and were therefore classified as positive. Next to this ELISA, a *Hin*FI DNA fingerprint was performed to test the diversity of the selected clones. All positive clones showed a similar *Hin*FI restriction pattern. This pointed towards a high degree of similarity between the selected clones, as was evidenced by sequence analysis of 11 clones. Three clones (1, 5 and 11), showed differences in their complementarity determining regions (CDRs) (Figure 1) and were therefore selected for further analysis.

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                  FR1                 CDR1       FR2             CDR2
Cl 1   AVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 7   AVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 4   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 9   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 2   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 6   QVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 3   DVQLVESGGGLVQAGGSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 8   DVQLVESGGGLVQAGDSLRLSCAASGRSFG SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 10  DVQLVESGGGLVQAGDSLRLSCAASGRTFS SYAMG WFRQAPGKEREFVA TISTLGGMTYYADSIKG
Cl 5   EVQLVESGGGLVQAGDSLRLSCADSGRTFS QYTMG WFRRAPGKEREFVA TISTLGGMTYYADSIKG
Cl 11  QVKLEESGGGLVQAGGSLRLSCAASGRTFS TYTMG WFRQAPGKERELVA AISWGGSRYYADSVEG

                  FR3                          CDR3          FR4
Cl 1   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 7   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 4   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 9   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTRVTVSS
Cl 2   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 6   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVST
Cl 3   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGIQVTVSS
Cl 8   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVSS
Cl 10  RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGTQVTVST
Cl 5   RFTISRDNAKNTVYLQMNSLKPEDTADYYCAA RFPGNGDYYLAPSYSY WGQGIQVTVSS
Cl 11  RFTISRDNAKNTVYLQMNSLKPEDTAVYYCAA KPPG-GIVTDTRKYDY WGQGTQVTVST
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Figure 1: Aligned amino acid sequence of the 11 KDEL-specific clones that were sequenced. Only clones 5 and 11 show differences in the complementarity determining regions (CDR) compared to the other clones.

Characterization of selected anti-KDEL VHH antibody fragments

To further evaluate these three clones, the performance of the different VHHs was tested in ELISA. A positive signal was only obtained at relatively high concentrations of antibody (higher than 150nM), which suggested a low affinity of the antibodies for the KDEL sequence. To increase the apparent affinity of the monoclonal VHHs, bivalent constructs were made and tested in ELISA. The bivalent clones (EC_{50} 15nM) performed approximately 100 times better than their monovalent (EC50 1.5μM) counterparts as is shown for clone 1 (Figure 2).

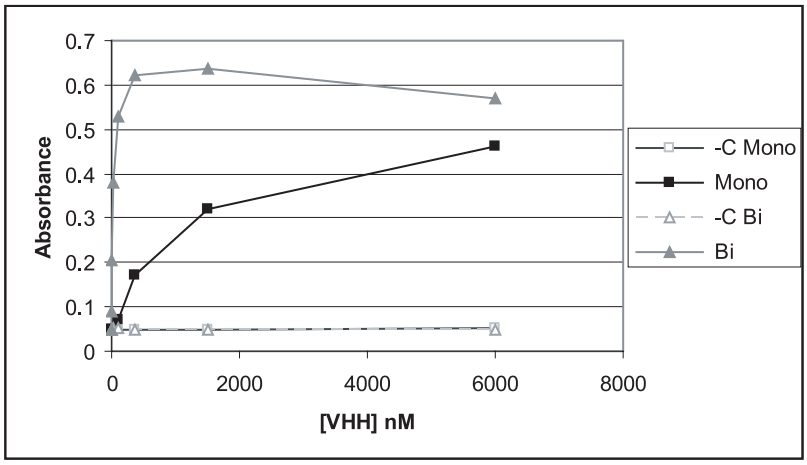

Figure 2: Effect of bivalency on the performance of the anti-KDEL VHH antibody fragments. An ELISA was performed on coated TropC-KDEL, detected with decreasing amounts of monovalent (EC_{50} 1.5μM) and bivalent (EC_{50} 15nM) clone 1 anti-KDEL. As negative control, a non-specific VHH was used.

To test the bivalent VHHs for their binding to ER resident KDEL-containing proteins in cellular extracts, they were first tested on Western blot of a HeLa cell lysate. Separate lanes of the blot were incubated with decreasing concentrations of each clone (Figure 3). All VHH clones detected several proteins in the HeLa extract and the observed band pattern at the highest concentration of antibody (1500nM) showed many similarities between the different clones, indicating that the different VHHs recognized the same proteins.

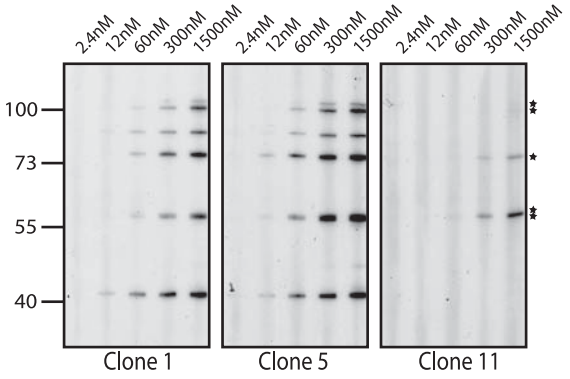

Figure 3: Comparison of the performance of the bivalent anti-KDEL clone 1, 5 and 11 on Western blot containing a HeLa cell lysate. The molar concentrations of the VHHs used for detection are depicted. Proteins detected by all three clones are indicated (asterix).

Protein	Acc.nr.	Mw	Function
Endoplasmin	P14625	92kD	Molecular chaperone
Putative alpha-Mannosidase C1 orf22	Q9BZQ6	100kD	
GRP78/BiP	P11021	72kD	Molecular chaperone
Calreticulin	P27797	48kD	Molecular chaperone
PDI A1	P07237	57kD	Catalyzes S-S bond rearrangement
PDI A6	Q15084	48kD	Catalyzes S-S bond rearrangement
ERp46	Q8NBS9	47kD	Possesses thioredoxin activity

Table 2: ER-resident proteins containing a C-terminal KDEL sequence. SWISSprot accession numbers (Acc.nr.), molecular weight of the unprocessed precursor (Mw) and function of the proteins are indicated.

Some of the detected proteins had a molecular mass corresponding to that of known ER resident proteins containing a C-terminal KDEL sequence, such as endoplasmin, putative alpha-mannosidase C1 orf22, GRP78, calreticulin, PDI A1, PDI A6, and ERp46 (Table 2). However, with decreasing amounts of antibody, a clear difference in the performance of the different VHH clones was observed (Figure 3). The bivalent construct of clone 11 did not detect the same number of proteins as clone 1 and 5. Furthermore, higher antibody concentrations were needed to obtain an equivalent signal with clone 11 compared to the other clones, indicating a lower affinity for the KDEL epitope. Based on these results, clone 5 was designated as the clone that performed best in this application and was therefore used for further experiments.

Proof of antigen specificity

To show that clone 5 recognized the KDEL sequence when it is present at the C-terminus of a protein, a competition experiment was performed. Bivalent VHH clone 5 was used to stain a Western blot of a HeLa cell lysate in the presence of the peptides KDEL, LEDK and KDELG (synthesized by Pepscan, Lelystad, The Netherlands) (Figure 4). With increasing amounts of the KDEL peptide, a clear reduction in the signal was observed. In contrast, when the peptides LEDK or KDELG were added, no competition was observed. The latter observation indicated that the VHH only recognized the KDEL sequence when present at the C-terminus of a peptide or protein. This was supported by ELISA experiments using proteins and peptides containing internal KDEL sequences, in which no positive signal was obtained with any of these antigens (data not shown).

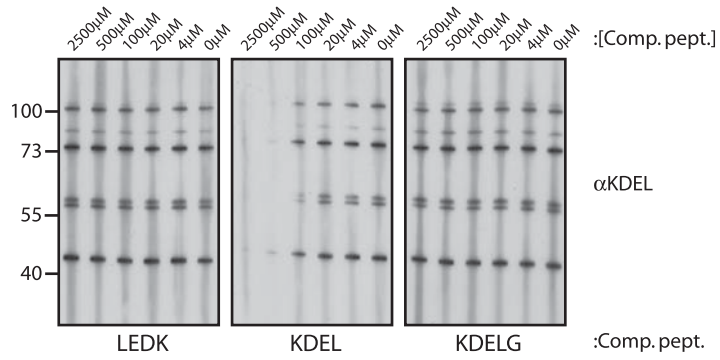

Figure 4: Specificity of the selected VHH clone 5 for the C-terminal KDEL sequence. During incubation of the anti-KDEL antibody at a 500nM concentration, increasing amounts of soluble peptide (LEDK, KDEL or KDELG) was added to different lanes to compete for the antigens present on blot.

To further confirm the specificity of the anti-KDEL VHH clone 5, immunoprecipitation (IP) experiments were performed with commercially available antibodies against known KDEL-containing proteins, i.e. anti-calreticulin, anti-PDI and anti-KDEL. The latter antibody has been shown to detect only the KDEL containing proteins endoplasmin and GRP78. Precipitated proteins were detected with the bivalent anti-KDEL VHH clone 5 (Figure 5). These data show that the proteins detected by the anti-KDEL VHH in a HeLa cell lysate indeed are the ER resident proteins endoplasmin, GRP78, protein disulfide isomerase (PDI) and Calreticulin.

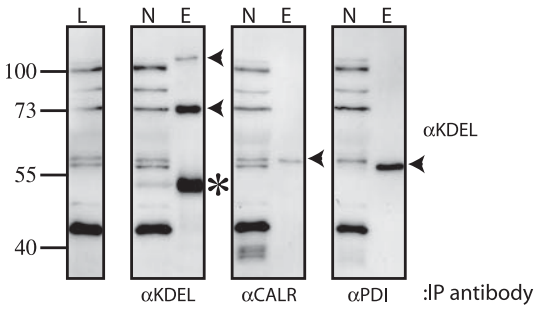

Figure 5: The anti-KDEL VHH recognizes the ER resident proteins endoplasmin, GRP78, PDI and Calreticulin. Commercially available antibodies against these known KDEL containing ER resident proteins were used to precipitate proteins from a HeLa cell lysate. The lysate (L), non-bound (N) and bound fraction (E) were analyzed on immuno blot with the bivalent anti-KDEL clone 5 VHH, after immuno-precipitation with the respective antibodies. The arrowheads indicate the precipitated proteins and the asterix indicates the heavy chain of the antibody used for the IP.

To identify the four additional proteins detected on Western blot, biotinylated bivalent clone 5 was used to purify the proteins from a HeLa cell lysate. The precipitated product was analyzed on SDS-page and on Western blot with the bivalent anti-KDEL clone 5 (Figure 6). The protein bands that corresponded to the molecular weight of the bands obtained on Western blot were excised from gel, trypsin digested and used for mass spectrometry (MS). The proteins endoplasmin (ionscore 7929), GRP78 (ionscore 5495) and PDI A1 (ionscore 2266) were positively identified, corroborating the results obtained with the commercial antibodies directed to known KDEL-containing proteins. Furthermore, the second lowest band consisted of the two KDEL proteins PDI A6 (ionscore 3830) and thioredoxin domain-containing protein 5 (ERp46) (ionscore 508), whereas the lowest band contained two proteins with a RDEL and not a KDEL retention signal. The most prominent protein found in the latter was collagen-binding protein 2 (colligin 2)(ionscore 1385), but also thioredoxin domain-containing protein 4 (Erp44) was found (ionscore 222). The results for the two remaining bands were inconclusive. However, judging from its molecular weight, one of the bands could be the putative alpha manosidase (table 2). From these data, it was concluded that the anti-KDEL VHH recognizes both KDEL and RDEL containing proteins.

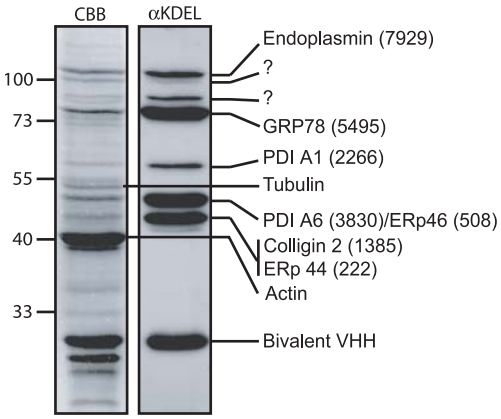

Figure 6: Characterization of the antigens recognized by the anti-KDEL clone 5 VHH. Target proteins were precipitated with the biotinylated bivalent VHH and analyzed on a coomassie (CBB) stained PVDF membrane. The CBB stained protein bands that corresponded to the signal obtained with the anti-KDEL antibody were identified by MS. Ionscores for the KDEL containing proteins are indicated between brackets.

Next, immuno fluorescence (IF) experiments were performed to further demonstrate that the VHH clone 5 recognized proteins in the ER. Labelling of HeLa cells with the bivalent anti-KDEL VHH showed almost identical labelling compared to that obtained with an antibody against the well-known ER marker PDI (Figure 7). This further proves that this antibody recognises specific proteins in the ER and illustrates its use in IF.

Finally, the anti-KDEL VHH was tested on lysates of cells from different mammalian species to investigate whether this antibody can be used to detect ER resident proteins in a wide variety of species (Figure 8). In all species tested (man, mouse, chimp and hamster) the band pattern of recognized proteins was similar, with the exception of one protein that was only detected in COS-1 and HeLa having a MW of approximately 90kDa. A comparison of the VHH antibody with a commercially available monoclonal anti-KDEL antibody (Stressgen, Victoria, Canada) showed that the VHH antibody recognized more proteins on Western blot (Figure 8).

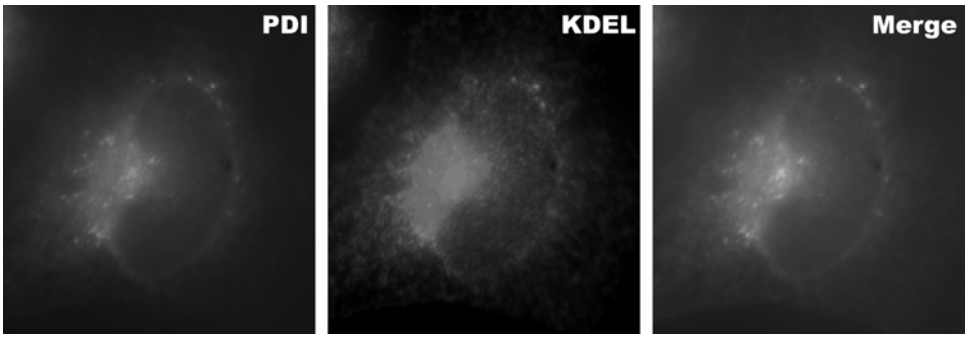

Figure 7: Immuno fluorescence staining of HeLa cells labeled with an anti-PDI antibody and the bivalent anti-KDEL clone 5 VHH. The merged picture clearly shows that both antibodies recognize antigens that co-localize in the ER.

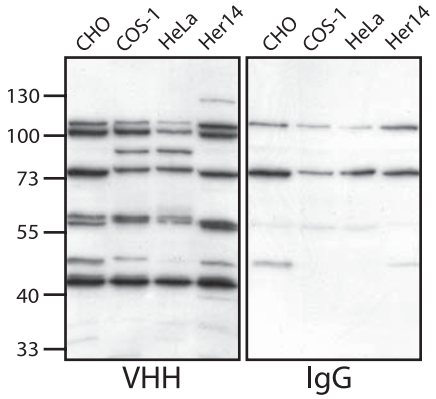

Figure 8: The anti-KDEL clone 5 VHH has wide species specificity. The performance of the bivalent VHH clone 5 (VHH) was analyzed and compared to a commercial anti-KDEL antibody (IgG) on a blot containing lysates of hamster (CHO), chimpanzee (COS-1), human (HeLa) and murine (Her14) cells.

Monitoring the ER-stress response with the KDEL-specific VHH

To show the applicability of this antibody in studies of ER stress, the ER response of HeLa cells was monitored after induction of stress by treatment with several different chemical agents. These agents, dithio-threitol (DTT), hydrogen peroxide (H_2O_2) and tunicamycin (TM) are known to cause an

unfolded protein response (UPR) at the level of transcription (Pakula et al., 2003). First, the viability of HeLa cells was estimated after incubation with different concentrations of these stress-inducing agents for 24 hours. The highest concentration of stressor that resulted in minimal cell loss after 24 hours was then used for subsequent experiments, which was 1.5mM DTT, 50μM H_2O_2 or 10μg/ml tunicamycin.

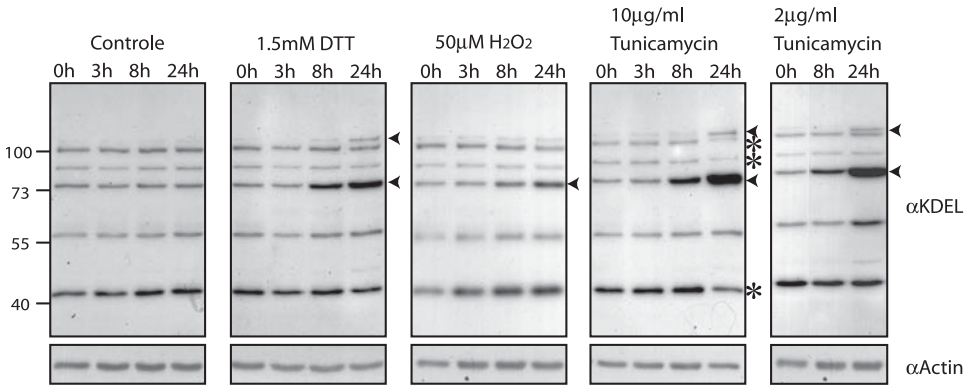

Figure 9: Differential ER-resident protein expression during ER stress induced with several different chemical agents and visualized by the anti-KDEL clone 5 VHH. Immuno blot with bivalent VHH clone 5 anti-KDEL. Cells were grown for 24 hours and subjected to the different stress inducers DTT (1.5mM), H_2O_2 (50μM) and tunicamycin (10μg/ml and 2μg/ml). Samples were taken after the indicated time periods. Proteins that are up- (arrowheads) or down-regulated (asterix) are indicated.

Cells were stressed for 24 hours with the determined concentrations of stress inducing agents and samples were taken at time points 0, 3, 8 and 24 hours. Cell lysates were prepared and samples were analyzed by Western blot with the bivalent anti-KDEL VHH clone 5 (Figure 9). This clearly showed that during treatment with the different stress inducing agents, especially GRP78 was up-regulated at the protein level. Furthermore, endoplasmin showed an up-regulation during treatment of the cells with DTT and Tunicamycin, but hardly after treatment with H_2O_2. The bands corresponding to the proteins PDI A1 and calreticulin showed a slight up-regulation with all three stress inducing agents. A striking observation was that some proteins also recognized by clone 5 anti-KDEL showed an apparent down-regulation after 24 hours treatment with tunicamycin. These results clearly show that upon ER stress, especially GRP78 shows a clear up-regulation at the protein level, whereas other ER

resident proteins show hardly any up-regulation or even a down-regulation. Furthermore, there is a clear difference in response upon treatment with different stressors, wherein tunicamycin treatment results in the most severe response after 24 hours. These differences were less apparent when tunicamycin concentrations were reduced to 2μg/ml, resulting in comparable differences as observed after the DTT treatment (Figure 9), which suggests that the variation between the effects of the different stressors is caused by the extent of ER stress

Discussion

To obtain KDEL-specific VHH antibody fragments that would be useful in the study of differential ER-resident protein expression, a large Llama-derived non-immune VHH library was used. Two *E. coli* expressed heterologous proteins, tagged with the KDEL sequence at their C-terminus, were used in two consecutive selection rounds. Of three different selected VHHs, the VHH that performed best in Western blot was subsequently thoroughly tested for its specificity and use in several biomolecular applications such as ELISA, Western blot, immuno fluorescence and immuno precipitation. Furthermore, the successful application of this antibody was shown in an ER stress model.

After selection and screening for anti-KDEL VHHs, the clones that were positive in phage ELISA were further evaluated. ELISA with soluble antibody did not result in comparable signals obtained by phage ELISA, in which the signal is amplified by the HRP-conjugated anti-M13 (anti-pVIII coat protein) antibody. Only very high concentrations of antibody yielded positive signals, which could be due to low antibody affinity. To increase the apparent affinity of the VHHs, bivalent constructs were made, which performed much better in ELISA than the monovalent constructs (Figure 2). This shows that, although selection using a non-immune phage display library may result in antibodies with relatively low affinity, the single domain character of the VHH allows the easy and quick modulation of the apparent affinity by means of simple cloning steps.

The high sequence similarity between the selected KDEL specific clones 1-10 indicated that these clones had advantageous characteristics over clone 11 which differed in all CDR regions from the other clones. The superior performance of clone 1 and 5 in Western blot, where a clear difference in performance was observed with clone 11, suggests that this was caused by a higher affinity of these clones for the KDEL epitope (Figure 3). Competition experiments with the peptides KDELG and KDEL (Figure 4) suggested that, at least clone 5, recognized only the KDEL sequence when present at the C-terminus of a protein. This had to be an essential feature of this antibody, as numerous non-ER resident proteins contain an internal KDEL sequence. The C-terminal KDEL specificity was confirmed by identification of the bands obtained on Western blot with clone 5 in two separate experiments; an IP experiment

with commercial antibodies against known KDEL containing proteins (Figure 5), and MS of precipitated proteins in an IP sample obtained with clone 5 (Figure 6). All these identified proteins were confirmed to be ER resident proteins containing a C-terminal KDEL or RDEL sequence by searching the SWISSprot database. The latter finding may be explained by the high degree of similarity between the charge of the amino acids arginine (R) and lysine (K). Indeed, the selected VHHs could also bind to C-terminal HDEL containing proteins in phage ELISA, although the signal was very low, suggesting a very low affinity for this epitope (data not shown). In the IP fraction obtained with clone 5, several other proteins were seen that did not correspond to the signal obtained on western blot. One band was especially prone and was identified as actin. Also another cytoskeleton protein was identified, tubulin (Figure 6). As ER resident proteins can bind a wide range of different proteins, e.g. via hydrophobic patches, co-immuno precipitation of proteins that are normally not exposed to the proteins that reside in the ER could be expected, since these proteins come into contact with each other during the IP protocol.

The selection of an antibody from a non-immune phage display library that specifically recognizes a four amino acid epitope, only when present at the C-terminus of a protein, is remarkable. Especially, since the average epitope recognized by an antibody is composed of more amino acids. Comparison of the bivalent version of this antibody with a commercially available anti-KDEL antibody, which was obtained with classical hybridoma technology, showed the superior performance of the selected antibody fragment (Figure 8).

In various ER stress models, using HeLa cells, we showed the successful applicability of the clone 5 VHH in cellular studies. We showed a clear increase of the proteins GRP78 and endoplasmin after treatment with the thiol reducing agent DTT and the N-glycosylation inhibitor tunicamycin (Figure 9). The up-regulation of GRP78 was less apparent upon induction of oxidative stress with H_2O_2 and there was no up-regulation observed of endoplasmin after this treatment. This clearly shows a different reaction of HeLa cells to the different forms of ER stress. There are two obvious explanations for these differences: It could either be that stress induced by different stress inducers results in different stress specific responses of the ER in regulating ER resident protein expression levels or the observed differences are caused by differences in the

extent of ER stress. The results obtained after treatment of cells with lower concentrations of tunicamycin (Figure 9) support the latter, since this resulted in comparable protein expression differences as observed after DTT treatment. This suggests that the up-regulation of the ER resident proteins endoplasmin and especially GRP78 at the protein level may be a measure for the severity of ER stress. In our experiments, H_2O_2 is less effective in inducing ER stress than tunicamycin at the concentrations tested. This is probably caused by different specificities of the used stress inducers. As H_2O_2 damages a wide variety of cellular components (i.e. DNA, lipids and proteins), treatment of cells with this agent results in cell death before a severe ER stress response is induced. In contrast, tunicamycin primarily influences mechanisms important for ER functionality (i.e. N-glycosylation), which allows treatment of cells with concentrations that are more effective in inducing an ER stress response.
Another intriguing aspect of these results is that some KDEL containing proteins are not affected in terms of protein expression while others show an apparent decrease in expression during severe ER stress (Figure 9). In contrast, it has been shown that several of these proteins are up-regulated at the transcriptional level during an UPR (Arvas et al., 2006; Kozutsumi et al., 1988; Martinez and Chrispeels, 2003). This illustrates the complex nature of the UPR, where the balance between transcription, translation and degradation determines the overall expression levels of a protein. Apparently, during expansion of the ER upon induction of stress, the ratio between different ER resident proteins shifts. The impressive up-regulation of GRP78 exemplifies its important role in maintaining ER homeostasis and its role in the control of the UPR (Bertolotti et al., 2000; Okamura et al., 2000; Shen et al., 2002). This up-regulation could be a negative feedback mechanism for the UPR and inhibit the induction of apoptosis while trying to compensate for the decreased folding capacity of the ER. The anti-KDEL antibody will allow the analysis to what extend the observed differential protein expression is conserved within different cell types and upon treatment with other stress inducers like thapsigargin, which induces ER Ca^{2+} depletion. It has been suggested that ER stress caused by ER Ca^{2+} depletion does not necessarily trigger the same signalling pathways as non-glycosylated proteins do (McCormick et al., 1997).

Our results clearly show that it is possible to select an antibody against a conserved epitope of only four amino acids and that this antibody can be

successfully applied in studies of ER homeostasis regulation. The application could be further extended to other ER stress studies as in ER storage diseases. The existence of several other biological mechanisms that utilize conserved epitopes for a specific function, such as the nuclear localization sequence (NLS) or the peroxisomal targeting sequence, could offer a target for similar selection experiments as has been done for protein modifications as phosphorylation, and glycation (Gronborg et al., 2002; Maguire et al., 2002; Richter et al., 2005). This would offer a useful tool in examining protein expression levels of specialized protein groups in a cell. The application of VHH antibody fragments combined with phage display techniques offer useful tools to be applied in this field.

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