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THE UNFOLDED-PROTEIN-RESPONSE ELEMENT DISCRIMINATES MISFOLDING INDUCED BY DIFFERENT MUTANT PRO-SEQUENCES OF YEAST CARBOXYPEPTIDASE Y

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The C-terminal region of the chaperone-like pro-sequence (p_Y) of yeast carboxypeptidase Y (CPY) is suggested to be crucial for the folding of mature CPY. In order to study the influence of hydrophobic residues in this domain, a set of mutations have been introduced in p_Y. Unexpectedly, only small amounts of CPY precursors are expressed when Leu108, at the C-terminal end of p_Y, is substituted for polar residues Lys, Arg or Asp. In contrast, substitution with hydrophobic residues Val, Ile or Ala permit normal expression. Interestingly, the poorly expressed molecules are core-glycosylated, implying that they have failed to leave the endoplasmic reticulum (ER). The ER-retained molecules cause an induction in the levels of BiP, signifying that polar substitutions at position 108 of pre-p_Y-CPY induce misfolding. Quite surprisingly, a reporter gene, linked to concatamerized unfolded-protein-response elements, reveals that p_Y-mediated misfolding of CPY is not really identical in all mutants. This shows that a simple transcriptional assay can assess the subtleties of pro-sequence mediated protein folding in an eukaryotic cell. © 1995 Academic Press, Inc.

Proteins are targeted to the secretory pathway with the help of 15 to 30 amino acid long N-terminal signals [1]. They consist of a hydrophobic core and are usually cleaved during translocation of the nascent protein across the membrane of the endoplasmic reticulum (ER) [2]. In many cases, the resulting polypeptide is not the mature molecule but an inactive precursor. The intermediates contain stretches of amino acids which do not belong to the mature protein. The peptides, constituting these amino acids, are referred to as pro-sequences. An active protein is obtained after the pro-peptide is enzymatically removed either in the Golgi or in the vacuoles [3, 4].

The pro-peptides are known to play an important role in the transport of precursor proteins in the cellular secretory pathway. Recent reports, which indicate that pro-peptides might be necessary for the correct folding of nascent proteins, have classified some pro-sequences as intramolecular

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Abbreviations: CEN, yeast centromere; CPY, yeast carboxypeptidase Y; ER, endoplasmic reticulum; *KAR2*, the yeast gene encoding BiP; *lacZ*, gene encoding *E. coli* ß-galactosidase; M_r, molecular mass; *PRC1*, gene encoding *S. cerevisiae* carboxypeptidase Y; p_Y, pro-sequence of CPY; UAS, upstream activation sequence; UPR, unfolded-protein-response; *wt*, wild type.

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chaperones [5]. The pro-sequence (p_y) of the *Saccharomyces cerevisiae* carboxypeptidase Y (CPY), covalently attached to mature CPY, is essential for proper *in vitro* folding of the precursor [6]. The yeast *PRC1* gene encodes the CPY precursor pre-p_y-CPY [7]. The 20-amino acid pre- or signal peptide is removed during translocation into the ER. The 91-amino acid p_y remains attached to CPY until it reaches the vacuoles. The mature CPY protein contains four N-glycosylation sites (i.e. Asn-Xaa-Ser/Thr motifs) [8]. Like all eukaryotic glycosylated proteins, the 512 amino acid p_y-CPY precursor undergoes core sugar additions in the ER, yielding the 67 kDa p1 form of p_y-CPY [9]. Further glycosylation in the Golgi produces the 69 kDa p2 form. Its distinct molecular mass (M_r) suggests that no more than 18-20 mannoses are attached to each of the four core oligosaccharide chains during the passage of p1 through the Golgi [9]. This is unusual amongst secreted glycoproteins, which normally undergo complex and extensive modification in the Golgi apparatus [8]. After traversing the Golgi, the p2 form of p_y-CPY is sorted to the vacuoles where active CPY is formed after p_y has been removed with the aid of the vacuolar proteases yscA and yscB [4].

Small deletions in the C-terminus of p_y have a drastic effect on the folding of CPY and prevent p_y-CPY to be exported from the ER [10], indicating that this region might contain structural elements which is essential for CPY activity. We set out to inquire if specific hydrophobic amino acids in the C-terminal part of p_y contribute to the folding of mature CPY. Residues at the junction of a pro-peptide and mature protein are normally solvent-exposed and should have no influence on protein folding. Unexpectedly however, we find that Leu108 at the C-terminal end of p_y (maturation of CPY occurs between Asn111 and Lys112) is important for correct folding of CPY. Mere replacement of this residue with a charged amino acid causes precursors to accumulate in the ER. Most likely, these mutant p_y-CPY proteins are misfolded since they cause the induction of the *KAR2* gene product BiP [11]. Remarkably, the unfolded-protein-response (UPR) element (an upstream activation sequence from the *KAR2* promoter) not only corroborates that the mutants are misfolded, but it also differentiates between the 'levels' of misfolding in the mutants. We believe that the ability to monitor subtle distinctions in protein folding in the ER would be an asset for further studies on the different pro-sequences associated with growth-factors, hormones and proteases [3, 4].

MATERIALS AND METHODS

Strains: All newly constructed plasmids were transformed in *Escherichia coli* HB101. Yeast transformations were performed [12] in *S. cerevisiae* strains W3116 (*MATa leu2-3 leu2-112 ura3-52 his3 prc1-D::HIS3 pep4-D1137*) and W3116-3xlacZ. W3116 was initially constructed from JHRY20-2CΔ3 [7] by gene replacement of *PEP4* (the gene encoding the protease yscA) using pJW1137 [13] and was kindly provided by Dr. J. Winther, Carlsberg Laboratory, Denmark. The genesis of the strain W3116-3xlacZ, a derivative of W3116, is described below.

DNA and plasmid constructions: The *ClaI-HindIII* fragment of the wild type (*wt*) *PRC1* gene (containing the promoter, coding sequence of pre-p_y-CPY and transcription terminator) from pLV9 [7] was converted to a *SalI-SacI* fragment for convenient cloning in *S. cerevisiae-E. coli* shuttle vectors. The plasmid pDC1 (encoding *wt PRC1*) was obtained by subcloning the *SalI-SacI* fragment of *wt PRC1* [7] in pDP83, a yeast centromere (CEN) vector. The mutations, at the C-terminus of the p_y peptide, were generated by PCR-mediated site-directed mutagenesis [14] on a 157 bp *MunI-BamHI* fragment from wild type *wt PRC1* [7]. The 5'-end primers for the PCR-s were the following,

5'-ACGCAATTGAAAACTATCAGaagCGTGTCAACAACAAGATTAAGG-3' (for Mut1: L108K); 5'-ACGCAATTGAAAACTATCAGagaCGTGTCAACAACAAGATTAAGG-3' (for Mut2: L108R); 5'-ACGCAATTGAAAACTATCAGgacCGTGTCAACAACAAGATTAAGG-3' (for Mut3: L108D); 5'-ACGCAATTGAAAACTATCAGgttCGTGTCAACAACAAGATTAAGG-3' (for Mut4: L108V); 5'-ACGCAATTGAAAACTATCAGattCGTGTCAACAACAAGATTAAGG-3' (for Mut5: L108I); 5'-ACGCAATTGAAAACTATCAGgctCGTGTCAACAACAAGATTAAGG-3' (for Mut6: L108A). The letters in lower casing denote the mutations. The 3'-end PCR primer was always 5'-GACCGGATCCTTTGCAGGATCGTTTCT-3' (it spans the *BamHI* site in *PRC1*). The mutations in the PCR products were confirmed by sequencing, using the Applied Bio-Systems 370A automated DNA sequencer. No other changes were introduced except the desired ones.

The 1003 bp *SalI-MunI* fragment (from pDC1) and the mutated *MunI-BamHI* fragments were subcloned in pUC19. The resulting plasmids contained a 1159 bp *SalI-BamHI* fragment (a 5' segment of *wt PRC1*) with mutations at the 3'-end of the pro-sequence. The complete mutant genes, encoding the promoter, coding sequence and terminator, were assembled as *SalI-SacI* fragments from the *SalI-BamHI* fragment (containing one of the six mutated pro-sequences of *PRC1*), the 931 bp *BamHI-NcoI* and the 546 bp *NcoI-SacI* fragments from pDC1. Plasmids pDC8, pDC9, pDC10, pDC20, pDC21 and pDC22 (see Table 1) were obtained by subcloning the above *SalI-SacI* fragments in the multi-copy 2μ-vector pDP34 [15]. pDC6 encodes the *wt PRC1*.

Construction of a vector for integration of a *lacZ* expression cassette, under the control of a promoter with three unfolded-protein-response elements, into the yeast chromosome: The plasmid pPFY7 is an integrative plasmid which contains the *LEU2* gene as the yeast selection marker. It lacks the upstream activation sequences (UAS-s) of the yeast *CYC1* promoter and consists of the *XhoI-BamHI* fragment of pLG670-Z [16]. The truncated *CYC1* core promoter is fused to *lacZ*, the gene for *E. coli* ß-galactosidase. The 22 bp unfolded-protein-response (UPR) element [17] was chemically synthesized as double-stranded DNA linkers, 5'-TCGAGGGAACTGGACAGCGTGTCGAAA-3' and 5'-TCGATTTCGACACGCTGTCCAGTTCCC-3'. These contain *XhoI* sticky ends on each strand, one of which is maintained as an *XhoI* site after subcloning in the XhoI site of pPFY7. The correct orientation of the UPR element, upstream of the *CYC1* core promoter, was confirmed by sequencing. The plasmid was named pCS2-1. Plasmids pCS2-2 and pCS2-3, encoding 2 and 3 copies of the UPR elements, were obtained by consecutive ligations of the UPR element to pCS2-1. The plasmid pCS2-3 (encoding 3 UPR elements upstream of *CYC1p-lacZ*) was integrated into the *LEU2* locus of the yeast strain W3116 by electroporation [12]. Correct gene integration and gene replacement events were verified by PCR. The resultant strain was named W3116-3xlacZ.

Immunoblot analysis, pulse-labeling and Northern blotting: For expression of CPY, two transformants from each of the transformations (listed in Table 1) were grown for 24 h in YPD [1% bacto-yeast extract (Difco), 2% bacto-peptone (Difco), 2% glucose and 0.05% bovine serum albumin (Sigma Cat. No. A-7638)]. Western/ Northern blotting and analysis of proteins after immunoprecipitation were performed according to protocols described earlier [18, 21]. Proteins were detected using the CPY-specific antiserum [19] and the Kar2-antibodies [20].

ß-galactosidase assay: Yeast cells were grown in YPD to an A_{600} of ~10. 10-20 μl cells were permeabilized for measurement of ß-galactosidase activity [21], which is expressed as arbitrary units after normalizing to cell culture density. The activity in the control strain, harbouring the plasmid pDC6 (encoding *wt PRC1*), was set to the value 1.

RESULTS AND DISCUSSION

PRC1 alleles encoding the mutations L108K (Mut1), L108R (Mut2) and L108D (Mut3) fail to express from a CEN-plasmid which is in stark contrast to the unmistakable expression of the *wt* gene

(unpublished observations). Pulse-chase experiments show that mutant p_y-CPY proteins are quickly degraded (results not shown). If degradation were to be the sole problem, it could be that this is redressed by over-expressing the mutated *PRC1* alleles. Like others [22], we have noticed a perceptible increase in the levels of expression of *wt*p_y-CPY when 2μ multi-copy plasmids are substituted for the single-copy CEN-vectors. After harvesting cells grown in liquid cultures, intracellular proteins from the strains yDC6, yDC8, yDC9 and yDC10 (bearing the genes which code for *wt*, Mut1, Mut2 and Mut3 *PRC1* respectively; Table 1) were analyzed by immune-blotting. It reveals that amounts of CPY-like proteins produced in the strains bearing Mut1, Mut2 or Mut3 are relatively small compared to the strain expressing *wt*p_y-CPY (i.e yDC6; see Fig. 1A). The mutant molecules have a smaller molecular mass (M_r) than the p2 form of p_y-CPY and are larger in size than mature CPY, hinting that they might accumulate as the p1 form of p_y-CPY [7, 9]. The observed disparity, in the levels of expressed *wt* and mutant proteins, do not reflect the amounts of CPY transcripts obtained from these strains (Fig. 1B). Attempts at introducing a radioactive label during biosynthesis of the mutant proteins showed that the mutants do not label as efficiently as the *wt* molecule (Fig. 1C; probably the mutants do not immunoprecipitate as efficiently as the *wt* protein). Moreover, pulse-chase experiments show that the mutant proteins are particularly prone to proteolysis, implying that they might be incorrectly folded (manuscript in preparation).

Experiments involving deglycosylation should reveal subtle differences in the length of *wt* and mutant polypeptides. Tunicamycin is a potent inhibitor of dolichol-phosphate:N-acetylglucosamine transferase which catalyses the first step in the biosynthesis of N-linked oligosaccharides [8]. Digestion of a glycoprotein with endoglycosydase F removes nearly all the sugar residues in an asparagine-linked oligosaccharide, excepting the one (i.e. an N-acetylglucosamine) which is directly attached to asparagine [8]. Expression of the mutants from cells (yDC6, yDC8, yDC9 and yDC10) treated with tunicamycin shows that the proteins are indeed N-glycosylated (Fig. 2A). The similarity of the banding pattern of the deglycosylated proteins hint that the four glycosylation sites in CPY are also occupied in the mutants as in the *wt* molecule. Treatment of total intracellular proteins with endoglycosydase F also confirms that mutant p_y-CPY molecules are glycosylated (Fig. 2B). It seems that there is no clear difference in M_r between deglycosylated *wt* and

Table 1. Plasmids and strains used in this study. The *wt PRC1* gene [7] encodes the unmutated p_y-CPY. The sequences Mut1, Mut2, Mut3, Mut20, Mut21 and Mut22, which encode the mutant p_y pro-peptides of CPY, are described in Materials and Methods. CPY is expressed from the multi-copy 2μ-plasmid pDP34 [15], encoding the different *PRC1* alleles, in the isogenic yeast strains W3116 (which is *pep4*) and W3116-3xlacZ (*3UPR-CYC1-lacZ* integrated in W3116; Materials and Methods).

Plasmid name	*PRC1* allele	Yeast strain(s) used for transformation	Corresponding yeast transformant(s)
pDC6	*wt PRC1*	(a) W3116 (b) W3116-3xlacZ	(a) yDC6 (b) 3xlacZ-*wt*PRC1
pDC8	Mut1 *PRC1*	(a) W3116 (b) W3116-3xlacZ	(a) yDC8 (b) 3xlacZ-Mut1PRC1
pDC9	Mut2 *PRC1*	(a) W3116 (b) W3116-3xlacZ	(a) yDC9 (b) 3xlacZ-Mut2PRC1
pDC10	Mut3 *PRC1*	(a) W3116 (b) W3116-3xlacZ	(a) yDC10 (b) 3xlacZ-Mut3PRC1
pDC20	Mut4 *PRC1*	(a) W3116 (b) W3116-3xlacZ	(a) yDC20 (b) 3xlacZ-Mut20PRC1
pDC21	Mut5 *PRC1*	(a) W3116 (b) W3116-3xlacZ	(a) yDC21 (b) 3xlacZ-Mut21PRC1
pDC22	Mut6 *PRC1*	(a) W3116 (b) W3116-3xlacZ	(a) yDC22 (b) 3xlacZ-Mut22PRC1

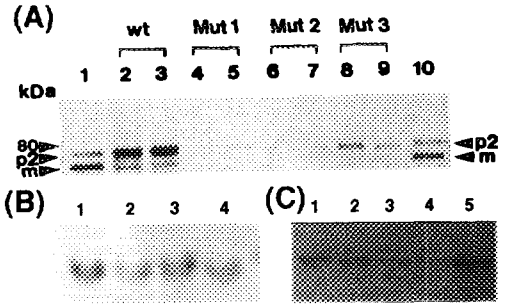

Fig. 1. Comparison of CPY-like proteins and CPY-specific mRNA from yeast strains harboring 2μ-plasmids. (A) Western blot analysis of intracellular proteins in *pep4* strains (Table 1). The lysates were prepared from ~80 A_{600} cells. Equivalent volumes (~12 μl) in 3x Laemmli buffer [18] were loaded onto a 10% SDS/polyacrylamide gel, except in lanes 1 and 10 where 2 μl was used. After fractionation and blotting, the transferred proteins were detected with anti-CPY serum [19]. Lanes 1 and 10, yDC6 (*wt*) plus 300 ng purified mature CPY (m; Sigma); lanes 2-3, yDC6 (*wt*; although it appears that *wt*p_y-CPY has undergone partial degradation in a *pep4* strain, we have not seen this consistently in all experiments); lanes 4-5, yDC8 (Mut1); lanes 6-7, yDC9 (Mut2); lanes 8-9, yDC10 (Mut3). The 80-kDa band used as marker belongs to the pre-stained low-molecular-mass standard proteins (Bio-Rad). 'p2' represents glycosylated p_y-CPY which has traversed the Golgi. **(B)** Northern blot analysis [21]. *PRC1*-specific mRNAs were detected in total RNA, extracted from yeast cells, with a DIG-labelled (Boehringer) 1113-bp *BamHI* fragment of PRC1 [7]. Lane 1, yDC6; lane 2, yDC8; lane 3, yDC9; lane 4, yDC10. **(C)** The proteins were pulse-labelled for 15 min and the CPY antigens were immunoprecipitated [18]. Different volumes of immunoprecipitates (resuspended in 40 μl of 3x Laemmli buffer) were loaded in the lanes. Lane 1, yDC6 (2 μl); lane 2, yDC8 (20 μl); lane 3, yDC9 (20 μl); lane 4, yDC10 (20 μl); lane 5, yDC6 (10 μl).

mutant proteins. These results suggest that the variation in M_r of the *wt* and mutant glycosylated proteins could be solely due to differences in the length of the oligosaccharide chains and not due to any heterogeneity of the polypeptide backbone. This would be possible if the mutant p_y-CPY molecules were to be core-glycosylated and be identical to the 67 kDa p1 form of *wt*p_y-CPY, the precursor known to be associated with the ER [9].

Suggestions have been made that the secondary structures formed at the C-terminal region of p_y are important for the formation of a compact folded structure, leading to the correct folding of p_y-CPY [23]. It is likely that efficient transport of the p_y-CPY zymogen is dependent on such a compact conformation. Our results support the notion that substitution of the C-terminal Leu108 by a polar residue has a profound effect which is deleterious for the folding of CPY, thus preventing precursor molecules from exiting the ER [24]. However, replacement of Leu108 by another hydrophobic residue (Val, Ile or Ala) permits precursors to traverse from the ER to the vacuoles where they form the p2 form of p_y-CPY (Fig 3A). Moreover, the mutants Mut4 (L108V), Mut5 (L108I) and Mut6 (L108A) do not seem to have any dramatic effect on the folding of CPY as measured by *in vitro* enzyme activity (data not shown) [19].

It could be assumed that during the process of achieving the compact structure, also known as the molten globule state (i.e. the postulated intermediate in the folding program), p_y constantly communicates with CPY through hydrophobic interactions. It is likely that the specific C-terminal hydrophobic residue Leu108 is vital for the folding of CPY. An unrelated protease subtilisin contains 'hydrophobic boxes' in its pro-region which are known to be essential for the function of the protease

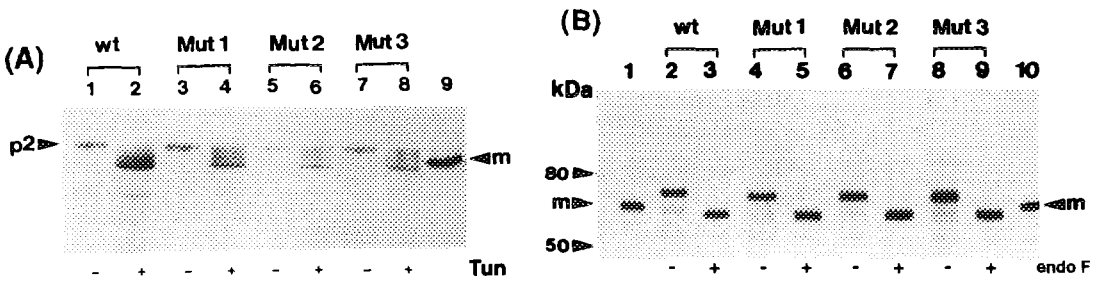

Fig. 2. Mutant p_y-CPY precursors are glycosylated. **(A)** Western blot analysis of proteins from tunicamycin treated *pep4* strains, as in Fig. 1. Equivalent volumes (~12 µl) in Laemmli buffer were loaded onto a 10% SDS/polyacrylamide gel, excepting for lanes 1 and 2 where 3 µl was used. After fractionation and blotting, the transferred proteins were detected with anti-CPY serum [19]. Lanes 1-2, yDC6 (*wt*); lanes 3-4, yDC8 (Mut1); lanes 5-6, yDC9 (Mut2); lanes 7-8, yDC10 (Mut3); lane 9, 300 ng purified, mature CPY (m, as in Fig. 2). 'p2' represents glycosylated p_y-CPY which has traversed the Golgi. **(B)** Western blot analysis after endoglycosidase F treatment [33] of proteins obtained from *pep4* strains. For the strains bearing the mutants, total intracellular proteins were concentrated 20-fold via TCA precipitation (before and after endoglycosidase treatment). Lanes 1 and 10, 300 ng purified, mature CPY (m, as in Fig. 1); lanes 2-3, yDC6 (*wt*); lanes 4-5, yDC8 (Mut1); lanes 6-7, yDC9 (Mut2); lanes 8-9, yDC10 (Mut3). The 50-kDa and 80-kDa bands used as markers belong to the pre-stained low-molecular-mass standard proteins (Bio-Rad).

[5, 25]. Replacement of residues in these boxes has an enormous impact on the folding of subtilisin. We believe that a similar hydrophobic interaction, crucial for the folding of CPY, has been disturbed serendepitously. This is remarkable, since published findings [10, 23] on the role of p_y in the folding of CPY would not have predicted these observations.

The immunoglobulin-binding protein BiP is known to be induced by malfolded proteins in the secretory pathway [26]. The regulation of BiP occurs both at the transcriptional and post-translational

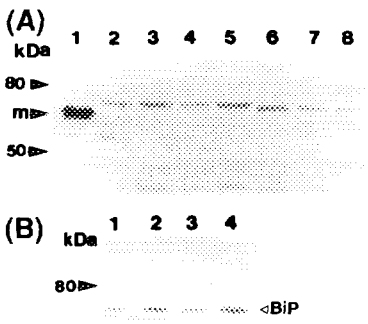

Fig. 3. Mutant p_y-CPY proteins, with a hydrophobic substitution at L108, do not accumulate in the ER and also do not induce BiP. The lysates were prepared from ~80 A_{600} cells as in Fig. 1. **(A)** Western blot analysis using CPY antibodies [19]. ~2 µl cell lysate was loaded in lanes 2-5 whereas 15 µl was loaded in lanes 6-8. Lane 1, 300 ng purified, mature CPY (m, as in Fig. 1); lane 2, yDC6 (*wt*); lane 3, yDC20 (Mut4); lane 4, yDC21 (Mut5); lane 5, yDC22 (Mut6); lane 6, yDC8 (Mut1); lane 7, yDC9 (Mut2); lane 8, yDC10 (Mut3). **(B)** Western blot analysis using Kar2 antibodies [20]. 20 µl lysate was loaded in each lane. Lane 1, yDC6; lane 2, yDC20; lane 3, yDC21; lane 4, yDC22. The 50-kDa and 80-kDa bands used as markers are identical to the ones in Fig. 2.

levels [11]. The *KAR2* gene is the BiP homolog in yeast [27, 28]. Initially, we attempted to monitor the induction of *KAR2* at the protein level (Fig. 4A). Total cellular proteins from the strains (yDC6, yDC8, yDC9, yDC10, yDC20, yDC21 and yDC22; Table 1), expressing *wt* and mutant p_Y-CPY proteins from 2μ-plasmids, have been probed with the Kar2p-antibody on a Western blot [20]. There is a distinct increment in the levels of BiP for strains expressing Mut1, Mut2 and Mut3 (Fig. 4A), whereas there is no such difference between the amounts of Kar2p in the strains expressing Mut4/ Mut5/ Mut6 and *wt* p_Y (Fig. 3B). The results palpably suggest that the Mut1p_Y-CPY, Mut2p_Y-CPY, Mut3p_Y-CPY proteins are misfolded.

We have attempted to quantify the degree of misfolding in the three mutant p_Y-CPY polypeptides Mut1, Mut2 and Mut3, using the unfolded-protein-response element (UPR) [17, 29]. The UPR is a 22 bp UAS in the *KAR2* promoter which has been found to be essential for gauging the presence of malfolded proteins in the ER [17, 30]. It seems that a single UPR element is capable of monitoring the global misfolding triggered by tunicamycin-mediated deglycosylation of all glycosylated proteins in the secretory pathway [17, 30]. The nature of the signals which mediate the transcriptional activation of *KAR2* in the nucleus is not yet fully understood [29].

In order to increase the sensitivity of the response to individual malfolded proteins, we have used upto three UPR elements (linked head-to-tail) and conjoined them to the yeast *CYC1* promoter which lacks its own UAS elements. The *E. coli lacZ* gene is under the control of this chimaeric promoter. To our great surprise we observe that the three mutant proteins can be distinguished from one another in the assay for ß-galactosidase activation. Mut3p_Y-CPY (coding for L108D) appears to be more misfolded (Fig. 4B) than the two mutant precursors coding for Mut1 (L108K) and Mut2 (L108R). This was hardly expected since the strains (3xlacZ-Mut1PRC1, 3xlacZ-Mut2PRC1 and 3xlacZ-Mut3PRC1), expressing the misfolded p_Y-CPY proteins (Table 1) manifest a similar induction of the *KAR2* mRNA (Fig. 4C; even quantification of the chemiluminescent bands in the Northern blot do not show any clear difference), relative to the strain 3xlacZ-*wt*PRC1 harboring *wt*p_Y-CPY.

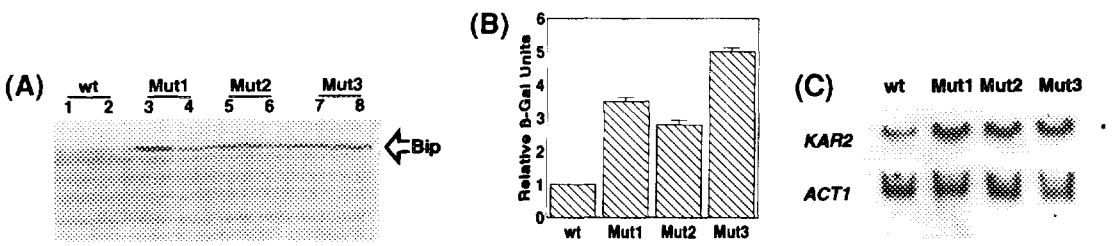

Fig. 4. Mutant p_Y-CPY proteins induce Kar2p (BiP), *3UPR-CYC1-lacZ* and *KAR2* mRNA. (A) Western blot analysis of intracellular proteins (see Fig. 1) by 10% SDS/PAGE. Equivalent volumes (~8 μl) in Laemmli buffer were loaded onto a 10% SDS/polyacrylamide gel. After fractionation and blotting, the transferred proteins were detected with anti-Kar2p serum [20]. Lanes 1-2, yDC6 (*wt*); lanes 3-4, yDC8 (Mut1); lanes 5-6, yDC9 (Mut2); lanes 7--8, yDC10 (Mut3). **(B)** Induction of ß-galactosidase in the strains 3xlacZ-*wt*PRC1, 3xlacZ-Mut1PRC1, 3xlacZ-Mut2PRC1, 3xlacZ-Mut3PRC1 (Table 1). The results described are an average of two independent experiments using three separate transformants. Each assay [21] is performed in duplicate and the ß-galactosidase units obtained from 3xlacZ-*wt*PRC1 are set to 1. **(C)** Northern blot analysis [21]. Total yeast RNA was probed with the DIG-labelled 677-bp *BglII* fragment of *KAR2* [27, 28] and the DIG-labelled ~1500 bp *BamHI-HindIII ACT1* (coding for yeast actin) [34], as in Fig. 1B. Lane 1, 3xlacZ-*wt*PRC1; lane 2, 3xlacZ-Mut1PRC1; lane 3, 3xlacZ-Mut2PRC1; lane 4, 3xlacZ-Mut3PRC1.

The work described here shows that one can discriminate between the levels of misfolding enforced upon a protein by marginally different chaperone-like pro-sequences. Indeed, we have also shown that levels of an active heterologous protein (i.e. human insulin-like growth factor-1) inversely correlates to the degree of misfolding induced by mutant pro-α-factor sequences (manuscript in preparation) [18]. We strongly believe that elucidation of multifaceted contacts, between a pro-sequence and its cognate protein during folding, would be facilitated with the help of this convenient colorometric assay [31, 32].

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