A novel yeast expression system for the overproduction of quality-controlled membrane proteins

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Received 24 June 2003; revised 30 July 2003; accepted 4 August 2003

First published online 18 September 2003

Edited by Irmgard Sinning

Abstract **Saturation of the cell's protein folding capacity and accumulation of inactive incompletely folded protein often accompanying the overexpression of membrane proteins (MPs) presents an obstacle to their efficient purification in a functional form for structural studies. We present a novel strategy for optimization of functional MP expression in *Saccharomyces cerevisiae*. This approach exploits the unfolded protein response (UPR) pathway, a stress signaling mechanism that senses the accumulation of unfolded proteins in the endoplasmic reticulum. We demonstrate that a high level of UPR induction upon expression of a MP reflects impaired functional expression of that protein. Tuning the expression level of the protein so as to avoid or minimize UPR induction results in its increased functional expression. UPR status can therefore serve as a proxy variable for the extent of impaired expression of a MP that may even be applicable in the absence of knowledge of the protein's biological function.**

Key words: Membrane protein; Protein overexpression; Protein folding; Unfolded protein response; *Saccharomyces cerevisiae*

1. Introduction

The production of properly folded membrane proteins (MPs) in the quantities required for structure determination is currently an enormous challenge. This situation reflects the limitations of existing systems for the overexpression of MPs of relatively low abundance [1,2]. Yeast-based expression systems show the greatest promise for the development of inexpensive alternatives to MP expression in *Escherichia coli*, particularly for medically important polytopic eukaryotic MPs, the overwhelming majority of which cannot be functionally overproduced in bacteria [1,3–5]. They are genetically flexible, suitable for large-scale fermentation, and unlike *E. coli*, perform some types of eukaryotic post-translational modifications [1,3]. However, their use has been greatly hampered because traditional expression protocols employing strong promoters and/or multicopy vectors often lead to saturation of the cell's protein folding capacity and accumulation of inactive unfolded or misfolded protein in intracellular membranes [6–10]. This situation complicates subsequent purification of functional protein as it often necessitates the inclusion in the purification scheme of a step based on the biological activity of the protein, such as ligand affinity chromatography. In the emerging field of MP structural genomics, such an approach to purification is untenable for the vast number of proteins for which functional information is lacking. In addition, failure to remove those molecules of a MP with non-native structures from a purified preparation that is to be used for 3D crystallization will result in their incorporation into a growing crystal, thus 'poisoning' the growth process, and so producing small (<50 μm), imperfectly ordered crystals with no or poor diffraction properties [11].

We set out to develop an improved expression system using the budding yeast, *Saccharomyces cerevisiae*, for MPs that reach their final cellular destination via the secretory pathway. Our aim was to exploit the unfolded protein response (UPR) pathway, a protein quality control mechanism operating in the endoplasmic reticulum (ER), to optimize the yields of correctly folded and targeted recombinant MPs. The UPR is an adaptive cellular mechanism that monitors the folding state of nascent polypeptides in the lumen of the ER, and during times of increased secretory and MP protein biosynthesis, adjusts the levels of luminal chaperones and folding enzymes accordingly. Proteins that fail to fold correctly, even after the folding capacity of the ER has been boosted, are degraded by pathways that are also regulated by the UPR, specifically, those targeting proteins to the vacuole and ER-associated degradation (ERAD) [12–14]. In yeast, activation of gene expression by the UPR (Fig. 1) is dependent on a transmembrane kinase, Ire1p, which resides in the ER. In the resting state, Ire1p is maintained in an inactive, monomeric form by binding to the ER luminal chaperone Kar2p, the yeast BiP homologue. Elevated levels of unfolded or misfolded proteins in the ER compete with Ire1p for binding to Kar2p freeing some Ire1p which dimerizes, undergoes *trans*-autophosphorylation, and initiates downstream signaling to the transcriptional apparatus [15]. Here we present a novel yeast expression system that combines tunable MP expression with an in vivo protein folding assay based on the UPR.

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00952-9

2. Materials and methods

2.1. Plasmid construction

All constructs were confirmed by dye terminator sequencing. Multicopy vectors for the expression of functional C-terminally tagged Ste2p and P2 (encoded by the *TeAT1* gene, GenBank accession number AJ278417) were constructed using a modified version of plasmid pYES260 [16] (obtained from EUROSCARF, Frankfurt, Germany). A DNA fragment containing a *Sma*I site and encoding both FLAG and His_6 tags was ligated into *Hin*dIII/*Not*I-digested pYES260. The *Xho*I site in the vector was then removed by *Xho*I digestion, T4 polymerase fill-in and religation to yield pYES2-FT.HT. A variant of pYES2-FT.HT was constructed by removal of a 484-bp *Pst*I fragment containing the entire *URA3* gene promoter (pYES2-URA3-d-FT.HT). The genes were cloned into pYES2-FT.HT by in vivo homologous recombination in yeast [17]. The *STE2* and *TeAT1* genes were amplified by polymerase chain reaction (PCR) from yeast genomic DNA (from strain S288C; Research Genetics, Huntsville, AL, USA) and plasmid pJL19, respectively. The PCR primers added 30 bp of sequence to the ends of the genes that are homologous to those flanking the *Sma*I site at the desired point of insertion between the *GAL1* promoter and the affinity tags. The *STE2* gene was cloned into pYES2-URA3-d-FT.HT in an identical manner (URA3-d-STE2p).

The tunable δ integration vector for P2 was constructed by excising the P2 expression cassette from the multicopy plasmid as a *Mlu*I/*Spe*I fragment, and blunt-end ligation into the blunted *Sac*I-digested pITy3 vector [18].

The UPR reporter plasmids were constructed from the pJS401 vector [19], which contains a transcriptionally silent *ICL1* promoter in front of *lacZ*, by integrating a fragment containing the UPR element (UPRE) into the *Bgl*II/*Sal*I site in the promoter. A variant of this construct carrying alternative selection markers was generated by introducing the *TRP1* gene at a unique *Hin*dIII site, and then replacing the *URA3* gene with the Tn*903 kan*^{r} marker from pITy3 by PCR-directed recombination.

2.2. Yeast transformation, growth and galactose induction

Transformation of strain BJ5464 with multicopy vectors was done by a yeast colony lithium acetate technique [20]. Expression cultures were grown in medium containing 0.67% yeast nitrogen base (YNB), 2% casamino acids, 4% raffinose, and buffered at pH 6.0 with 50 mM sodium phosphate. The δ strains were generated by transformation of BJ5464[*cir-0*] (cured of the 2-μm plasmid as described [21]) harboring pMEGA2-ΔURA3 [22] and a UPRE-*lacZ* plasmid with 5 μg of *Xho*I-digested P2 δ vector or pITy-3 (as control) using a high-efficiency lithium acetate method [23]. Transformants were selected on plates containing 5 mg/ml G418. For expression studies, δ strains were grown in 1% yeast extract, 2% bacto-peptone and 4% raffinose at 30°C and induced with 2% galactose at an OD_{600} of 1–2.

2.3. Southern blotting

P2 gene copy number was determined by Southern analysis as described [18]. Probe synthesis, hybridization and detection were performed with digoxigenin (DIG) system reagents according to the DIG application manual (Roche Applied Science, Mannheim, Germany). Exposed films, for which responses were in the linear range, were scanned using an Agfa Snapscan 600 flatbed scanner and densitometry performed with Scion Image Software (Scion Corporation, Release Beta 4.0.2). Copy number determinations were performed in triplicate.

2.4. Adenosine and histidine transport assays

Cells were harvested, washed three times with a medium containing 0.67% YNB and 2% galactose (MMG), and resuspended in the same medium to an OD_{600} of 6 and 40 for adenosine and histidine transport assays, respectively. Aliquots of cells (50 μl) were added to the same volume of MMG containing 2 μM [^{3}H]adenosine (NEN Life Science, Boston, MA, USA) or 1.2 mM L-[^{3}H]histidine (Amersham Pharmacia Biotech), and incubated for 10 and 2 min, respectively, at 22–24°C. Control experiments showed that the time intervals used were well within the initial linear phase of substrate accumulation curves and thus approximate initial rates of transport. Influx was terminated by the addition of 1 ml of ice-cold MMG (containing 1 mM adenosine in adenosine uptake assay) and filtration of the cell suspension under vacuum through 0.45-μm Whatman GF/F filters. The filters were washed once with 10 ml of the same ice-cold medium and the retained radioactivity was measured by liquid scintillation counting.

2.5. Preparation of membranes and total cell extracts

Total and plasma membranes were isolated as described [24,25]. Protein concentrations were determined by the Pierce BCA microassay (Perbio Science, Bonn, Germany) in the presence of 0.5% sodium dodecyl sulfate (SDS) using bovine serum albumin as standard. Total cell protein extracts were prepared as described [26].

2.6. SDS–polyacrylamide gel electrophoresis and immunoblotting

Expressed proteins were analyzed by electrophoresis through 8% Tricine–SDS polyacrylamide gels [27]. Ste2p and P2 were detected by Western blotting with a murine monoclonal anti-Penta-His antibody (Qiagen, Hilden, Germany) and the Western-*Star*™ chemiluminescent immunoblot detection kit (Applied Biosystems, Bedford, MA, USA). Immunodetection of the plasma membrane H^+-ATPase was done with a rabbit polyclonal anti-yeast H^+-ATPase antibody. Densitometry was performed on data collected within the linear response range for protein concentration and film exposure.

2.7. UPR induction

Assay of β-galactosidase (β-gal) was carried out as described [28] and the activity normalized to the protein concentration determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany).

3. Results

3.1. Expression of trypanosomal adenosine transporter activates UPR

To test how readily achievable UPR induction is upon the functional expression of plasma MPs in *S. cerevisiae*, we expressed the *S. cerevisiae* α-factor receptor (Ste2p) and the *Trypanosoma equiperdum* P2 H^+/adenosine cotransporter from a multicopy 2μ-based vector under the control of the *GAL1* promoter in cells carrying a sensitive reporter of UPR activation. The reporter construct consists of the β-gal gene (*lacZ*) driven by a single UPRE from the *KAR2* promoter

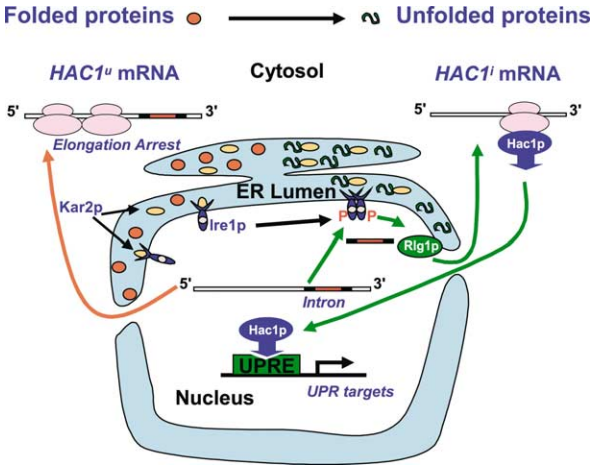

Fig. 1. The UPR signaling pathway in *S. cerevisiae*. Increased levels of unfolded/misfolded proteins in the ER decrease the levels of free Kar2p by binding to it. The occupancy of Kar2p bound to Ire1p is reduced and promotes Ire1p dimerization. Ire1p *trans*-autophosphorylates activating its nuclease domain. Through the action of Ire1p and tRNA ligase (Rlg1p), the constitutively expressed transcript for Hac1p (*HAC1*^{u}), the UPR-specific transcription factor, is processed by removing its intron, leading to Hac1p synthesis. Hac1p translocates to the nucleus, binds the UPR element (UPRE) of target genes, and induces their expression. Red arrow indicates processes occurring when UPR is off and green arrows when UPR is on.

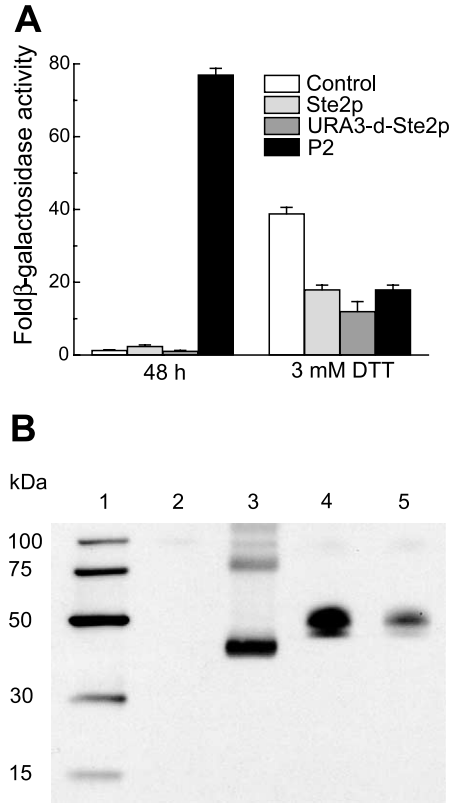

Fig. 2. Expression of P2 transporter activates UPR. A: β-Gal measurements on cells harboring a UPRE-*lacZ* plasmid and expressing Ste2p and P2 (C-terminally FLAG-His_6-tagged) from multicopy vectors at 48 h galactose induction. For DTT-treated samples, 3 mM DTT was added with galactose and activity measured after 4 h. URA3-d-Ste2p denotes a vector with increased copy number due to poor expression of the *URA3* marker [42]. Results expressed relative to zero galactose induction time values are means ± S.E.M. ($n = 3$–6). B: An immunoblot representative of two experiments prepared with total membranes from 4 h galactose-induced cultures. Lane 1, His_6-tagged ladder; lane 2, 20 μg control; lane 3, 20 μg P2; lane 4, 1 μg URA3-d-Ste2p; lane 5, 1 μg Ste2p. The anti-His_6 antibody used to probe the blot showed no cross-reactivity with proteins in the control membranes, and specifically labeled P2 which migrated with an apparent molecular mass of 40 kDa (predicted molecular mass of 54 kDa including 3-kDa tags) and Ste2p which showed multiple bands in the range 46–56 kDa as previously reported [43].

(UPRE [29]; Fig. 1) on a multicopy vector. Fig. 2A shows that Ste2p expression had little effect on β-gal activity. A further ~2-fold increase in Ste2p expression achieved by using a version of the vector in which the promoter of the *URA3* selection marker was removed (URA3-d-STE2p) still failed to elicit a UPR (Fig. 2A).

In contrast, overexpression of the P2 transporter resulted in a greater than 70-fold increase in β-gal activity (Fig. 2A), although it was up to 20-fold less abundant in yeast total membranes than Ste2p (Fig. 2B). To test whether our inability to detect UPR induction upon expression of Ste2p might be due to impaired UPR functioning accompanying its higher level of expression, we examined the effect of Ste2p and P2 expression on a dithiothreitol (DTT)-induced UPR. DTT disrupts protein folding in the ER by preventing disulfide bond formation and is often used to induce the UPR [15]. DTT-induced responses in cells expressing P2 and Ste2p from the original 2μ vectors were of a similar magnitude suggesting that the absence of UPR induction upon expression of Ste2p is unlikely to be due to a compromised UPR (Fig. 2A). Surprisingly, expression of either protein resulted in DTT-induced responses that were < 50% of that seen in the control strain. This effect may result from an increased oxidation of the DTT. Recently it was shown that the proliferation of ER membranes occurring upon the impaired secretion of a heterologous modified fungal cutinase resulted in oxidative stress, and as a consequence, the carbonylation of cellular proteins [30].

3.2. A novel expression strategy

To determine whether the observed UPR induction upon expression of the P2 adenosine transporter reflects its impaired functional expression, the expression system depicted in Fig. 3 was constructed. This system allows sampling of a range of P2 synthesis levels above and below that at which the UPR is induced. In order to avoid the occurrence of a transcriptional limit imposed by the low cellular levels of the galactose-responsive transcriptional switch proteins (Gal4p-Gal80p-Gal3p) in cells carrying multicopies of the *GAL1* promoter, Gal4p, Gal80p and Gal3p were overexpressed from the pMEGA2-ΔURA3 plasmid [22]. The P2 expression cassette from the 2μ vector was subcloned into the tunable δ integration vector [18]. This vector carries the bacterial NEO kanamycin resistance gene and results in stable integration of multiple gene copies into the Ty δ sequences of *S. cerevisiae* chromosomal DNA (Fig. 3). In general, the NEO-conferred geneticin resistance ($G418^R$) of a transformant increases monotonically with increasing δ vector copy number, thus allowing the tuning of gene copy number by varying the geneticin concentration used for transformant selection [18]. A protease-deficient strain, BJ5464[*cir-0*], harboring pMEGA2-ΔURA3 and a UPRE-*lacZ* reporter plasmid, was transformed with the P2 δ vector.

3.3. Strong activation of UPR reflects impaired P2 expression

We determined P2 gene copy number, β-gal activities, initial rates of [^{3}H]adenosine influx into intact cells, and P2 expression in total cell protein extracts, for eight transformants that were designated w1, w2, lb1, lb2, lb3, db1, db2, db3 (δ strains), and compared these results with those obtained with the 2μ strain (2μ-P2) under identical growth conditions (Fig. 4). P2 gene copy number was determined by Southern

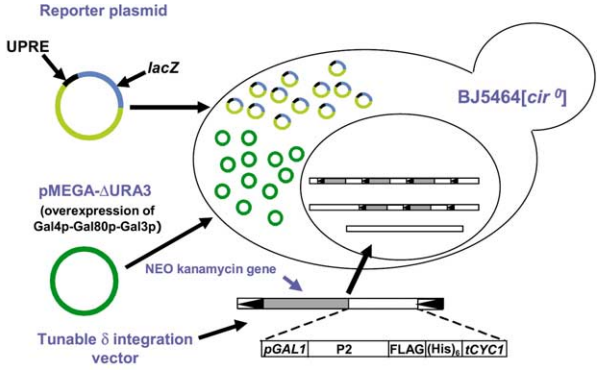

Fig. 3. A novel yeast expression system with in vivo protein folding assay based on the UPR. Boxed black triangles in the tunable δ integration vector represent δ sequences which target chromosomal integration of the vector by homologous recombination to the 150–200 copies of the Ty δ sequence present on yeast chromosomes [18].

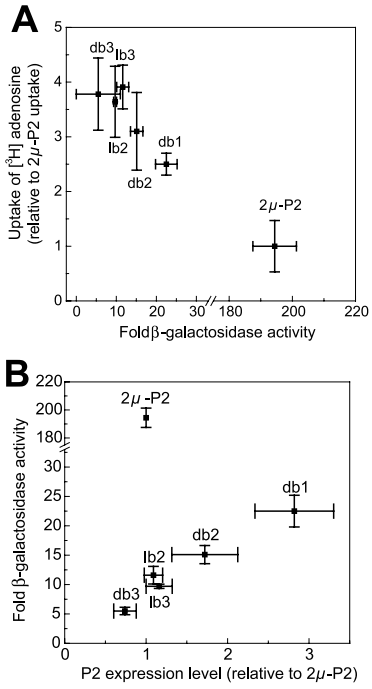

Fig. 4. Strong activation of UPR reflects impaired P2 expression. A: Inverse correlation between the initial rates of 1 μM [^{3}H]adenosine uptake into intact cells (at 4 h induction) and the degree of UPR induction (β-gal activity measured at 48 h induction). B: Correlation between degree of UPR induction and the level of P2 protein expression. Expression levels were determined by immunoblotting of total cell protein extracts from 4 h galactose-induced cultures and are expressed relative to that in 2μ-P2. Results are means ± S.E.M. ($n = 3$).

analysis of total yeast DNA. Surprisingly, although the P2 gene was detectable in genomic DNA from strains w1, w2 and lb1, P2 expression in these strains could not be detected in immunoblots or adenosine uptake experiments (data not shown). The reason that these strains failed to express P2 is presently unclear. P2 gene copy numbers in the remaining strains were 45 ± 5, 13 ± 3, 7 ± 1, 20 ± 2, 10 ± 2 and 12 ± 1, for 2μ-P2, lb2, lb3, db1, db2 and db3, respectively.

Strikingly, for those strains in which P2 expression could be detected in immunoblots, cell surface expression of the transporter, as assessed by initial rates of adenosine transport after 4 h of galactose induction, was inversely related to the degree of UPR induction (Fig. 4A). β-Gal activities were measured after 48 h of galactose induction to allow accumulation of this long-lived enzyme (half-life in yeast ~20 h [31]), and thus detection of low levels of UPR activation. The degree of UPR induction was directly related to the level of P2 protein expression except in strain 2μ-P2 (Fig. 4B). The difference in P2 protein abundance between strains does not account for the differences in their transport activity. For instance, the amount of expressed P2 in cell extracts of 2μ-P2, lb2 and lb3 was similar, although the rate of adenosine transport was four-fold lower for 2μ-P2 than for lb2 and lb3. Although the abundance of P2 protein does not clearly correlate with P2 gene copy number in the δ strains, db1 with the highest copy number does show the highest P2 expression level. The 2μ-P2 strain with an ~2-fold higher copy number than db1, surprisingly, showed ~3-fold lower P2 levels. These results taken together suggest that the synthesis of the P2 transporter in strain 2μ-P2 occurs at a rate that overwhelms the ER's folding machinery, strongly activating the UPR, and leading to its increased degradation via the ERAD pathway.

To examine whether increased transport activity in the δ strains was due to improved surface expression of functional P2 protein, transporter abundance in purified plasma membranes from lb3 and 2μ-P2 was examined (Fig. 5A). Unexpectedly, the average improvement in transporter expression was only 1.3 ± 0.3-fold ($n = 4$). This modest increase does not readily explain the ~4-fold difference in adenosine transport activity shown by these strains (Fig. 4A). One explanation for the discrepancy might be that in 2μ-P2, one or more components of the quality control apparatus have been saturated allowing inactive unfolded/misfolded P2 transporters to escape from the ER, as has been suggested to occur upon overexpression of the ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator [32].

A remarkable difference between membranes from lb3 and 2μ-P2, revealed on inspection of Coomassie-stained gels (data not shown), is the decreased abundance of a band with an apparent molecular mass of 100 kDa in lb3 membranes. This protein was identified as the plasma membrane H^+-ATPase in immunoblots and was ~4-fold less abundant in lb3 membranes than in control membranes (Fig. 5B). Its expression in 2μ-P2 was only slightly reduced (~1.2-fold). The H^+-ATPase generates the electrochemical proton gradient that provides the driving force for nutrient uptake by H^+-dependent cotransporters [33,34]. Reducing H^+-ATPase expression has been shown to result in decreased amino acid transport [35]. These results suggest that the driving force for H^+-coupled adenosine transport is likely to be reduced in the δ strains compared to 2μ-P2 under these conditions, and thus the improvement in functional expression of P2 in the δ strains, as assessed by adenosine uptake (Fig. 4A), is probably underestimated. Strong support for this conclusion comes from the observation that initial rates of endogenous H^+-coupled histidine transport under identical conditions were significantly lower in lb3 and db1 compared to 2μ-P2 (27 ± 1, 20 ± 2 and 69 ± 3 pmol/10^7 cells/min, respectively, means ± S.E.M., $n = 3$). Additional evidence in favor of this interpretation of these data is provided by experiments examining P2 expression as a function of the galactose induction time (Fig. 6). The levels

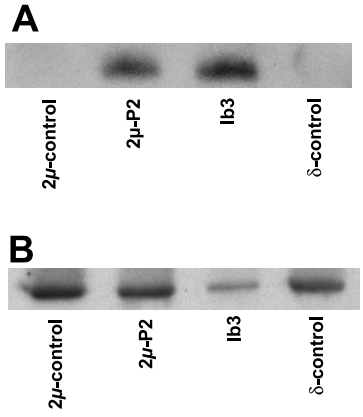

Fig. 5. Comparison of P2 levels in purified plasma membranes from 2μ-P2 and lb3. Five μg of membranes prepared from 4 h galactose-induced cultures were analyzed. A: Immunodetection of P2. B: Immunoblot probed with anti-yeast H^+-ATPase antibody. Results are representative of three independent experiments.

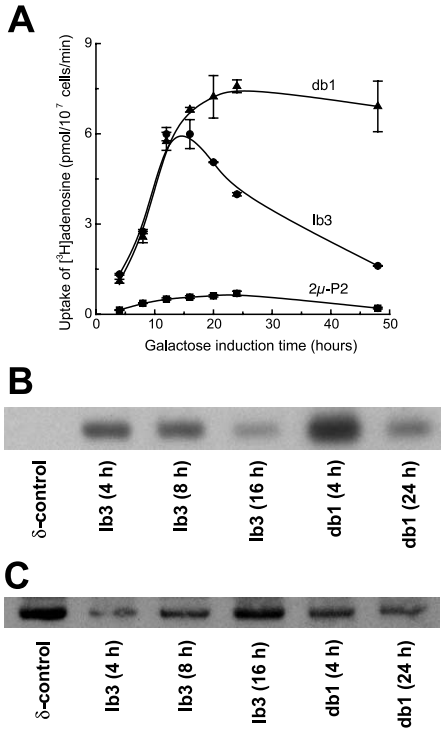

Fig. 6. P2 expression as a function of galactose induction time. A: Initial rates of 1 μM [^{3}H]adenosine uptake as a function of induction time interval for 2μ-P2, lb3 and db1. B: Immunoblot analysis of P2 expression in plasma membranes (5 μg) from these strains at various induction times. C: Immunoblot analysis of H^+-ATPase expression in plasma membranes (5 μg) at various induction times. Results are representative of two independent experiments.

of P2 protein in plasma membranes from lb3 and db1 at different induction times (Fig. 6B) do not readily account for the observed changes in transport activity (Fig. 6A). An explanation for this apparent anomaly is suggested by the unexpected finding that in lb3, expression of the H^+-ATPase recovered from its ~4-fold reduced level at 4 h to near normal levels at 16 h (Fig. 6C). Therefore, peak expression of functional P2 in these strains occurs at around 4 h and appears to coincide with the lowest levels of H^+-ATPase expression. Changes in transport activities thereafter reflect alterations in both P2 and H^+-ATPase cell surface abundance. Strain db1 showed the highest level of P2 expression (Fig. 6B: 3–5% of plasma membrane protein as estimated by comparison of the immunoblot signal with that obtained from known amounts of the 53 kDa His_6-tagged protein, Positope™ (data not shown)) and will be used for future purification and crystallization work.

4. Discussion

One of the most vexing problems facing a structural genomics approach to MPs is the inadequate supply of purified properly folded MPs. This problem provided the motivation for the development of a novel strategy for MP overexpression in *S. cerevisiae* that exploits a cellular protein quality control mechanism, the UPR, to assess the productive folding of recombinant MPs in vivo. This approach allowed us to demonstrate that for some plasma MPs, exemplified by the P2 adenosine transporter, the use of traditional expression methods employing strong promoters and multicopy vectors may lead to saturation of the ER's protein folding apparatus and their impaired expression. Reducing synthesis rates for such proteins to a level that better matches the ER's folding capacity may result in their improved functional expression, as found here for the P2 transporter. We have found that two human G protein-coupled receptors (GPCRs) that are functionally expressed in *S. cerevisiae* induce the UPR to a similar extent as the P2 transporter (D.A. Griffith, unpublished results), suggesting that the functional expression of these proteins may also be improved by our approach. Further experiments are currently under way. Homologous overexpression of the α-factor receptor did not activate the UPR suggesting that this protein does not encounter severe problems in the folding of its ER lumen-exposed domains. We have also found that the overproduction of several plant solute transporters in *S. cerevisiae* has little effect on the activity of the UPR pathway (D.A. Griffith, unpublished results). These results taken together suggest that the strategy we have developed may be most applicable to MPs from organisms which are distantly related in evolutionary terms to *S. cerevisiae*. These are exactly the proteins which have proved to be the most difficult to efficiently express in this yeast [1].

An additional advantage of our approach is that optimization of functional expression of a protein occurs in a single step with the isolation of transformants showing low levels of UPR induction. This obviates the need to perform time-consuming studies on the effects of reduced cultivation temperature and modified growth media on the protein's functional expression.

Previously reported systems for monitoring in vivo folding of recombinant proteins were developed with *E. coli* and rely on a 'folding reporter' protein that indicates the extent of productive folding of fused protein domains [36–38]. Aggregation of target proteins and their incorporation into inclusion bodies was correlated with reduced formation of the functional reporter proteins. It is unclear whether such an approach will work in yeasts which have a reduced tendency for inclusion body formation. These approaches have also thus far only been used to screen for proteins that fold efficiently in *E. coli*, and not, as has been done in this study, to optimize the expression of a single variant.

In the present work, we demonstrate for the first time that a protein quality control stress response, the UPR, can be exploited to optimize synthesis rates of a MP so as to better utilize the intrinsic protein folding capacity of the host cells. Our system has potential applications in addition to the provision of native membrane protein samples for structural studies. For instance, improved cell surface expression of human GPCRs in *S. cerevisiae* should facilitate the development of high-throughput screening assays for novel ligands [39–41].

Acknowledgements: We would like to thank Karl Wittrup for his kind gift of the plasmid pITy3, Hans-Joachim Schüller for the pJS401 plasmid, James Hopper for the pMEGA2-ΔURA3 vector and Ramón Serrano for the anti-yeast H^+-ATPase antibody. We would also like to thank Rita Wiemeyer for excellent technical assistance in many of the experiments.

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