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Regulated increase in folding capacity prevents unfolded protein stress in the ER

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Summary

Stimulation of thyrocytes with thyroid stimulating hormone (TSH) leads to a morphological change and a massive increase in thyroglobulin (Tg) production. Although Tg is a demanding client of the endoplasmic reticulum (ER), its increase did not result in significant accumulation of unfolded protein in the ER. Instead, ER chaperones and folding enzymes reached maximum synthesis rates immediately after TSH stimulation, before significant upregulation of Tg synthesis. The resulting increase in folding capacity before client protein production prevented cellular unfolded-protein stress, confirmed by the silence of the most conserved branch of the unfolded protein response. Thyrocytes set an example of physiological adaptation of cells to a future potentially stress-causing situation, which suggests a general strategy for both non-secretory and specialized secretory cells.

Key words: Protein folding, Folding stress, Endoplasmic reticulum, Unfolded protein response, UPR, Thyroglobulin, Chaperone

Introduction

Specialized secretory cells have a well-developed pathway for transport of proteins out of the cell. Proteins destined for secretion need to fold into their native structure in the endoplasmic reticulum (ER), the first compartment they encounter on their way out (Anelli and Sitia, 2008; Christis et al., 2008). To facilitate folding, the ER is filled with resident chaperones and folding enzymes, which are present in millimolar concentrations (Marquardt et al., 1993; Stevens and Argon, 1999). In constitutively secreting cells, pressure on the ER folding capacity is continuous, since folding is often the rate-limiting step in ER export and secretion (Lodish, 1988; Pelham, 1989; Petaja-Repo et al., 2000).

The ER changes its folding capacity according to need, for example during development, or upon changed needs for protein secretion in the adult body, such as by thyrocytes or B lymphocytes (Federovitch et al., 2005). When folding capacity is exceeded and unfolded or misfolded proteins accumulate in the ER lumen, a stress response pathway is activated, termed the unfolded protein response (UPR). The UPR involves two clearly distinguishable phases: the generation of the signal in the ER lumen, and the propagation of this signal across ER membrane and cytosol into the nucleus. The mammalian ER contains three main stress sensors: Ire1, ATF6 and PERK (Harding et al., 1999; Shi et al., 1998; Tirasophon et al., 1998; Wang et al., 1998; Yoshida et al., 1998). These proteins sense the unfolded protein signal in the ER, and their signaling leads to attenuation of protein synthesis, upregulation of folding capacity of the ER, and increase in degradation machinery (reviewed by Ron and Walter, 2007; Zhang and Kaufman, 2006).

A functional UPR is important for survival of secretory cells (Marciniak and Ron, 2006): mice lacking PERK, for instance, develop diabetes and exocrine pancreatic insufficiency caused by dysfunction of the ER (Harding et al., 2001). Although in a specialized secretory cell unfolded proteins do trigger the UPR to increase folding capacity, this signal is not necessary for every step of ER expansion. When resting B cells differentiate into antibody secreting plasma cells, initial expansion of the ER precedes increase of antibody synthesis (van Anken et al., 2003), demonstrating that upregulation of ER-resident proteins in these cells initially is controlled by a developmental program and is not due to unfolded protein stress per se. The ER expansion may be regulated by components of the UPR, in particular by some of the transcription factors, but their activation does not originate from and is independent of an unfolded protein stress signal in the ER lumen (van Anken et al., 2003; Hu et al., 2009). For full ER expansion, XBP-1 activation is essential as *XBP1*$^{-/-}$ lymphocytes fail to secrete immunoglobulins and to develop fully to plasma cells (Reimold et al., 2001; Tirosh et al., 2005).

Perhaps B lymphocytes are unique in their developmental program, as they are dedicated to a short burst of secretion activity, ended by apoptosis. The question is whether a cell type that constantly changes from ER expansion to reduction without death would instead use a mild stress response for its regulation (Rutkowski et al., 2006). We therefore analyzed how thyrocytes manage ER folding capacity. These cells produce thyroglobulin (Tg), the precursor of thyroid hormones T3 and T4. The level of Tg production by thyrocytes is controlled by thyroid stimulating hormone (TSH) (Kim and Arvan, 1993; Sargsyan et al., 2004; Szkudlinski et al., 2002). In stimulated thyrocytes as much as 10–20% of total protein synthesis is dedicated to Tg production (Kim and Arvan, 1993). This 330 kDa protein contains 10-15 *N*-linked glycans, forms 60 native disulfide bonds (Van de Graaf et al., 2001) and folds in large transient covalent aggregates (Kim and

Arvan, 1991). Tg needs simultaneous assistance of multiple ER-resident chaperones and folding enzymes to fold properly into a transport-competent dimer (Di Jeso et al., 2003; Kim and Arvan, 1991; Kim et al., 1992). Tg therefore can be considered a demanding client of the ER.

We compared cells grown in the absence of TSH with cells cultured for increasing times with hormones. We found that Tg was upregulated after a time lag, whereas total levels of resident ER proteins increased during the stimulation period, caused by a boost of their synthesis immediately after hormone addition. Tg synthesis was not yet upregulated at these times, and at no time point was XBP-1 spliced, indicating absence of an ER stress signal. eIF2α phosphorylation did not follow Tg expression either but instead showed an inverse correlation, confirming that early upregulation of folding capacity prevented accumulation of significant amounts of unfolded protein, thereby preventing ER stress, and allowing a more subtle and mild use of the UPR system for ER expansion. This physiological adaptation leading to upregulation of ER folding capacity via a developmental pathway is emerging as a general strategy for specialized secretory cells.

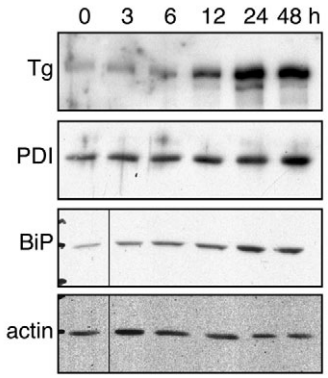

Fig. 1. TSH induced expression of thyroglobulin, BiP and PDI. FRTL5 cells were deprived of hormones for 5 days, after which they were stimulated by the addition of 6H for the indicated times. Cells were lysed and the indicated proteins were detected by immunoblotting. Gels were loaded with an equal amount of protein per lane. Actin is shown as a loading control. The activation experiment was repeated eight times in total. See supplementary material Fig. S1 for a quantification of these blots.

Results

Thyroglobulin is produced upon TSH stimulation

The immortalized thyroid cell-line FRTL5 is widely used to study Tg folding and transport (Kim and Arvan, 1993; Kuznetsov et al., 1994; Sargsyan et al., 2002). The cells have retained most of the features of differentiated follicular thyroid cells, such as TSH dependence for growth and Tg production (Ambesi-Impiombato et al., 1980). We confirmed, using western blot analysis, that after hormone deprivation for 5 days the Tg level was low and that expression was induced by re-addition of the hormone. The level of Tg started to increase after 12 hours of stimulation, reaching a maximum level after 24 hours of stimulation (Fig. 1 and supplementary material Fig. S1). This increase was not due to conformational selectivity of the antibody used as two different polyclonal antisera gave the same results (supplementary material Fig. S2). PDI and BiP (also known as GRP78) levels increased during the stimulation period as well (Fig. 1, and supplementary material Figs S1, S2). A concentration range of lysate confirmed that enhanced chemiluminescence (ECL) signals for PDI, BiP, actin and Tg correlated with amount of protein (supplementary material Fig. S3). We concluded that FRTL5 cells provided a suitable model system to follow the increase of ER folding capacity after hormonal stimulation. We chose to study the early response of thyrocytes to TSH stimulation because this is the period in which the cells are adapting from one state to the other: from low secretion rate to maximum Tg secretion. To avoid possible artifacts from cell splitting or from too low or high cell densities, we started experiments when cells were ~50% confluent, and stopped after 48 hours, just before trypsinization became necessary.

Levels of ER proteins increase upon stimulation of FRTL5 cells

Because of the upregulation of PDI and BiP upon hormonal stimulation of FRTL5 cells we set out to determine the expression pattern of a broader range of ER chaperones and folding enzymes by following their expression levels over time. We therefore separated post-nuclear lysates of FRTL5 cells using 2D gel electrophoresis before and after several periods of stimulation; Fig. 2A shows the 48-hour time point. Gels were silver-stained, spots quantified, and proteins identified by MALDI ToF mass spectrometry (supplementary material Table S1). We loaded 2D gels with equal amounts of protein per lane to ensure similar conditions in all samples during focusing and running of gels, and normalized expression levels to the total signal in the gel, thus showing a change relative to the total protein composition of the cells. We chose this way of normalization to allow for changes in volume of the cells, as discussed before (van Anken et al., 2003) and used it consistently throughout all experiments. Levels of most resident ER proteins were increased after 12 hours of stimulation and continued to increase during the stimulation period (Fig. 2B and supplementary material Fig. S4A). Although the patterns were not identical, all identified ER-resident proteins were upregulated during stimulation, reflecting the need for increased folding capacity because of the increase of Tg production.

Not all proteins are upregulated in response to TSH

During hormone deprivation FRTL5 cells slow down their cell cycle. Upon stimulation the cells not only increased Tg production but also returned to the same doubling time as before hormone deprivation [44 hours in our experiments, similar to the 38 hours reported by Medina and Santisteban (Medina and Santisteban, 2000)]. To test whether the upregulation of ER chaperones and folding enzymes was specific rather than resulting from a general increase in protein synthesis, we analyzed expression patterns of a broader range of proteins. Whereas some non-ER proteins increased similarly to the resident ER proteins, indicative of the significant remodeling these cells need to undergo, most non-ER proteins followed very distinct expression patterns. A few examples of non-ER proteins are shown in Fig. 2B. Because we had already identified the expression patterns of the larger ER-resident proteins we used 12.5% gels to concentrate our analysis away from very large proteins. Identified protein spots were quantified and proteins with similar regulation of expression were grouped in an unbiased way using the self organizing tree algorithm (SOTA). The program constructed a binary tree (dendrogram) in which the terminal nodes were the resulting clusters of proteins with similar expression patterns (Herrero et al., 2001). The clusters we found had expression patterns varying from gradual upregulation (cluster A) to relative

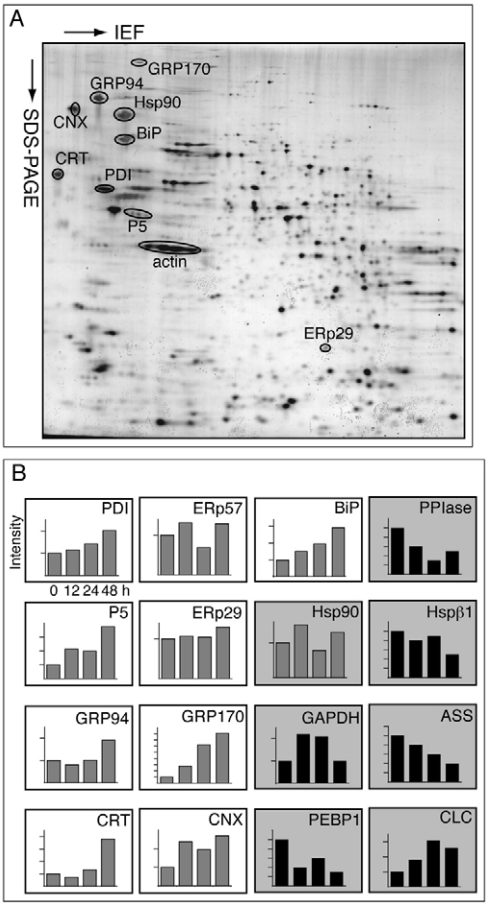

Fig. 2. ER-resident proteins are upregulated in response to TSH stimulation. FRTL5 cells were deprived of hormones for 5 days, after which they were stimulated by the addition of 6H. At the indicated stimulation times, cells were lysed, proteins were separated using 2D-gel electrophoresis, and gels were silver stained. (**A**) 2D gel of 48-hour-stimulated thyrocytes. The identity of some abundant proteins is indicated. (**B**) The gels were scanned and spot intensities quantified. Histograms show the expression patterns of selected proteins, normalized to their expression level at the 0-hour time point (see supplementary material Fig. S4A for these histograms displayed at the same scale to allow easier comparison of expression patterns between different proteins). Histograms on a white background are of ER-resident proteins. The histograms with gray bars show quantification of signals in the gel shown in A, and histograms with black bars show quantification of samples run on 12% gels (see also supplementary material Table S1). PPIase, 40 kDa peptidyl-prolyl *cis-trans* isomerase; ASS, adenosuccinate synthase; PEBP1, phosphatidylethanolamine binding protein 1; CLC, clathrin light chain A; CRT, calreticulin; CNX, calnexin.

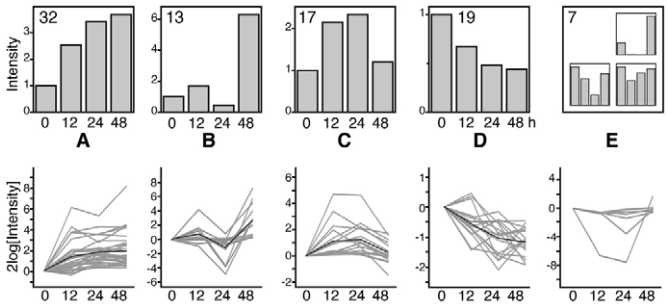

Fig. 3. Proteins form groups with distinct expression patterns. Protein spots from silver-stained 2D gels, as shown in Fig. 2, were quantified using the PDQuest software package and clustered with SOTA analysis. Histograms show the mean expression profile of proteins in each cluster. The cluster letter is indicated below each histogram, and the number of proteins in the cluster is indicated in the top left corner (see supplementary material Fig. S4B for these histograms displayed at the same scale to allow easier comparison of expression patterns between different clusters). The lower panels show the expression patterns of all proteins in each cluster, with the mean expression pattern as a black line. Note that the relative changes in expression level are indicated as change in intensity in the upper panel, and as the ^{2}log change in intensity in the lower panel. Proteins in cluster A are upregulated continuously during activation (examples include ERp29, PSMB4, PFDN2, LCAD). Proteins in cluster B show a lag time before large-scale upregulation (examples include APOA1, PSMA2, PRX3, Rab11b). Proteins in cluster C have a peak in their expression level during the time course investigated (examples include GPDH-C, RhoGDI1, RANBP1, myosin RLC-A), and proteins in cluster D are not upregulated, which leads to an apparent downregulation of their relative expression (examples include Hsp27, CYP40, Ran, Vps33). Cluster E contains proteins that did not fit in any of the clusters A-D (examples include PA28α, aldose reductase, stathmin, GAPDH). Identities of all clustered proteins and relative expression levels are presented in supplementary material Table S1.

downregulation (cluster D) (Fig. 3, supplementary material Fig. S4B and Table S1). Most proteins changed, but not all and not all to an equal extent, as illustrated by clusters D and E in Fig. 3 and supplementary material Fig. S4B. The different representation in the main text and supplementary figures allows the relative and absolute differences to be examined. We conclude that all ER-resident proteins show the same pattern: gradual upregulation in response to thyrocyte stimulation. The Tg-associating protein ERp29 (Sargsyan et al., 2002) indeed joined the other ER-resident proteins in cluster A, as expected, although its changes were minimal. The clustering of non-ER proteins within an expression group did not always correlate clearly with their biological function. Yet, almost all proteasome subunits we identified were upregulated during stimulation, and grouped with the ER proteins in cluster A, which is not unexpected as protein misfolding and degradation obligatorily accompany protein folding (Travers et al., 2000). We concluded that upon TSH stimulation thyrocytes remodeled in particular their secretory capacity to accommodate subsequent production of Tg.

Maximum translation of ER-resident proteins before Tg upregulation

Silver-stained gels only allow analysis of changes in steady-state protein levels, which dampens changes in synthesis or degradation. To follow changes in protein synthesis directly, we pulse-labeled thyrocytes before or after several periods of stimulation and analyzed equal amounts of protein from post-nuclear lysates using SDS-PAGE. Overall changes were evident and reproducible, but the pace of the process varied slightly from one experiment to the other. We therefore show here the results of a representative experiment. The total amount of label incorporated during the 30-minute pulse, which directly preceded cell lysis, increased ~4.5 times over 48 hours (Fig. 4A,B), reflecting the overall increase in protein synthesis, consistent with the silencing of eIF2α. We confirmed that the indicated band in Fig. 4A was Tg by both immunoprecipitation (not shown) and western blot analysis (Fig. 4A) and subsequently quantified Tg synthesis levels directly from

non-saturated scans of the radioactive signal of one-dimensional gels. We used one-dimensional gels for this analysis, as large proteins such as Tg do not behave well during isoelectric focusing.

We expressed the amount of newly synthesized Tg as a percentage of the Tg synthesized before stimulation (Fig. 4B), thus visualizing how the levels of protein synthesis in general, and those of Tg specifically, changed over time. We also expressed the amount of newly synthesized Tg as the percentage of total protein synthesis (Fig. 4C) to show, for every time point, how Tg synthesis related to total protein synthesis. At 0 hours, when the cell is in a resting state, protein synthesis is very low (Fig. 4A, Fig. 5B) and Tg synthesis contributes 6%. Upon activation, the contribution of Tg to total protein synthesis drops to 3.5%, which we interpreted as preparation of the cell for large-scale protein production, during which priority is given to synthesis of other proteins (including the ER chaperones) over Tg. The absolute rate of Tg synthesis did increase, however, such that at 6 hours after activation the Tg signal had increased slightly, and the increase in overall protein synthesis was much stronger. Closer to the new steady-state, after 48 hours, Tg synthesis represented more than 7% of total protein synthesis of the cell but synthesis of Tg was more than fivefold higher than at the resting state. This difference was due to the higher levels of total protein synthesis levels at 48 hours compared to 0 hours, as the cell is in a different metabolic state (e.g. it is dividing again after 48 hours, as described earlier).

Please note that, as mentioned above, the percentages in Fig. 4C are calculated for each individual time point, in contrast to Fig. 4B, which allows comparison *between* the different time points. We interpret the similarities between the 6% at 0 hours and the 7% at 48 hours (Tg synthesis relative to total protein synthesis at those time points) as an indication that after 48 hours of activation the cells have reached a new equilibrium in which they sustain massive Tg production.

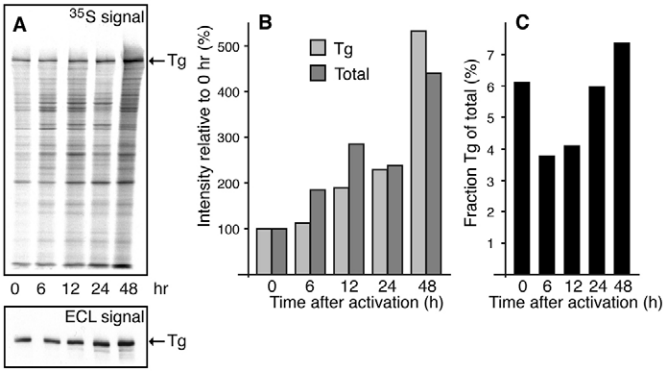

Fig. 4. General protein synthesis but not thyroglobulin expression increases early during stimulation. FRTL5 cells were deprived of hormones for 5 days, after which they were stimulated by the addition of 6H. At the indicated time points, cells were pulse-labeled for 30 minutes and lysed. Post-nuclear lysate, normalized to total amount of protein, was separated on 6% and 10% SDS-PA gels. (**A**) Autoradiograph of the 6% gel (upper panel; ^{35}S signal), and the corresponding Tg western blot (lower panel; ECL signal). The arrow indicates Tg. Total signal and Tg signal were quantified using the Image Quant software package using non-saturated exposures of 6% and 10% SDS-PA gels. (**B**) Histogram showing the mean values expressed as a change relative to label incorporation before stimulation for both total protein and Tg. (**C**) Percentage of total label incorporated into Tg after different stimulation times.

The lag between the stimulation signal and Tg synthesis, combined with the immediate increase in protein synthesis upon stimulation, suggests that synthesis of other proteins is needed before production of Tg can be increased efficiently. Likely candidates for these proteins are ER-resident chaperones and folding enzymes because their steady-state levels increased from the start. We therefore separated post-nuclear lysates of pulse-labeled thyrocytes using 2D gel electrophoresis, dried the gels, and quantified autoradiography signals (see supplementary material Table S2) of major spots, including ER-resident proteins. SOTA analysis of the synthesis patterns again yielded several distinct clusters (Fig. 5A). Because comparisons between steady-state levels and changes in kinetics do not necessarily correlate directly, we numbered these clusters to differentiate them from those shown in Fig. 3. Synthesis of most proteins was upregulated

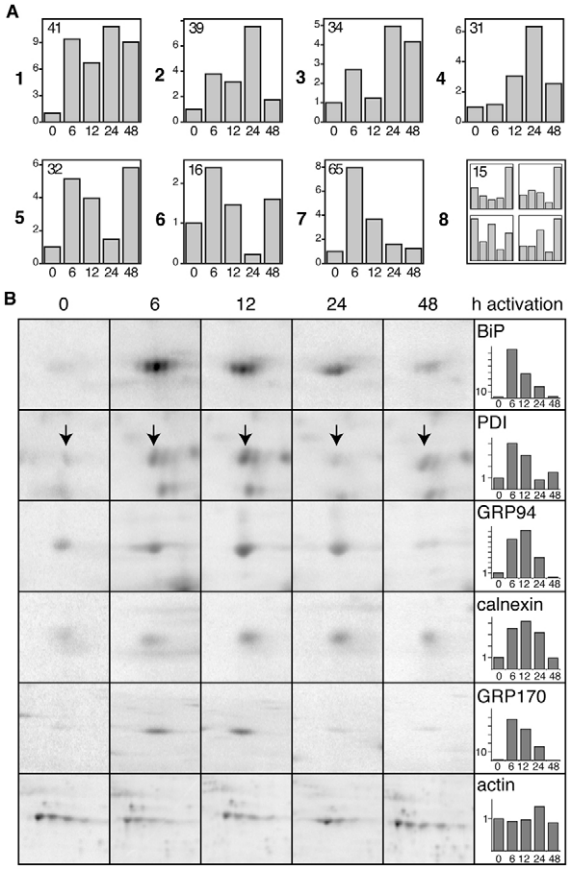

Fig. 5. Synthesis of ER-resident proteins increases early during stimulation. FRTL5 cells were deprived of hormones for 5 days, after which they were stimulated by the addition of 6H. At the indicated time points, cells were pulse-labeled for 30 minutes and lysed. Post-nuclear lysates, normalized to total amount of protein, were separated using 2D gel electrophoresis. The gels were dried and exposed to phosphorimaging screens. Radiolabeled spots were quantified and clustered using SOTA analysis as in Fig. 3. (**A**) Histograms show the mean expression profiles of proteins in each cluster. The cluster number is indicated on the left of the histograms, the number of proteins in the cluster is indicated in the top left corner of the histogram. Expression profiles of the individual spots are presented in supplementary material Table S2. (**B**) Excerpts of gels showing spots corresponding to ER-resident proteins (the PDI spot is indicated with an arrow) and actin are shown together with the histograms representing their relative synthesis levels at the indicated time points, normalized to an arbitrary unit of 1 at 0 hours.

after 6 hours of stimulation (clusters 1 and 5-7), whereas a subset of proteins was upregulated only after a lag (clusters 2-4). ER-resident proteins all grouped in cluster 7, with a strong peak in expression early after stimulation (Fig. 5A,B). For chaperones and folding enzymes the high rate of synthesis was not maintained throughout the stimulation period although most ER protein synthesis rates continued to exceed the rates before stimulation (see cluster 7 in Fig. 5A). The drop in synthesis rate (although they never dropped below the levels before stimulation) can be explained by the relatively long half-life of ER-resident proteins. A boost of synthesis hence provides increased folding capacity over a prolonged period of time. BiP and GRP170 showed the strongest increase in their synthesis level (see *y*-axes in Fig. 5B), although the fold increase may be overestimated because of the near absence of a detectable spot at the 0 h time point. Their strong increase is reflected in their steady-state levels, which also showed the strongest increase. Both GRP94 and calnexin synthesis levels peaked at 12 hours after stimulation, but were significant already after 6 hours. Synthesis of ER-resident proteins increased and reached maximum rates shortly after stimulation of thyrocytes, well before Tg synthesis increased. We concluded that the increase of folding capacity preceded production of the major ER client.

The apparent discrepancies between steady-state patterns and rate of synthesis patterns of individual proteins result from the intricate relationships between synthesis and degradation of both the proteins and the mRNA. Typically, the increase in steady-state lags behind an increase in synthesis, as seen when comparing Figs 2 and 5. We consider it likely that protein degradation rates changed as well, but as half-lives of the identified resident ER proteins are longer than 2 days, we could not detect any differences.

XBP-1 is not spliced during activation of thyrocytes

Our observation that synthesis of resident ER proteins increased before Tg synthesis suggests that this was regulated by a mechanism other than the classical UPR signal of folding protein overload in the ER. To confirm that upregulation of ER chaperones and folding factors was not caused by an increased folding load of other ER clients, we tested whether XBP-1 precursor mRNA was spliced shortly after stimulation. As a positive control for Ire1 activation at the time points we examined, we treated cells in parallel with tunicamycin, a potent UPR inducer. Before and at several time points after application of hormones, we isolated total RNA, reverse transcribed it, and amplified the XBP-1 cDNA using primers flanking the region that is spliced out by Ire1. At no time was XBP-1 spliced (Fig. 6A). This implied that the increase of chaperone production was not induced by unfolded protein stress in the ER lumen, even though Tg production was increased more than fivefold (Fig. 4B) (Sargsyan et al., 2004). Treatment with tunicamycin did result in detection of the spliced fragment at all time points (one of which is shown in Fig. 6A), establishing that the FRTL5 cells are capable of sensing and transducing ER stress. When we examined phosphorylation of eIF2α as another measure of UPR induction early after activation (Fig. 6B), we found the protein already phosphorylated before stimulation, most likely as a result of the hormone deprivation and consequent activation of alternative (i.e. non PERK) eIF2α targeting kinases. We detected only a small transient increase in phosphorylation above the basal level and a subsequent silencing of the involved kinases when Tg levels increased, consistent with the increase in total protein synthesis seen in Fig. 4A. This confirmed that PERK was not activated by the Tg increase. Similarly, we only detected CHOP (also known as DDIT-3 and GADD153) expression, as a measure of ER stress, after we treated cells with tunicamycin and not at any time during hormone deprivation or treatment (Fig. 6B). We concluded that ER expansion was not induced by an unfolded protein stress signal in its lumen, but that both client protein upregulation and ER remodeling were the direct result of the TSH-induced signaling cascade.

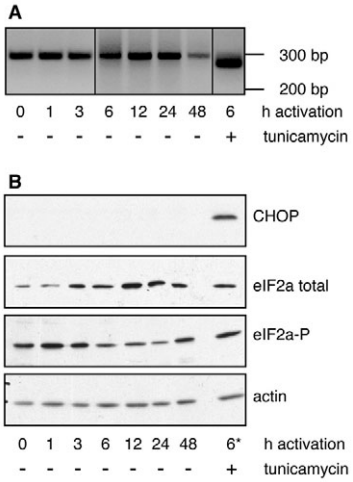

Fig. 6. Response of UPR components on TSH activation of thyrocytes. FRTL5 cells were kept without TSH for 5 days, after which they were stimulated by the addition of 6H. (**A**) Total RNA was isolated and after reverse transcription a PCR reaction was performed with XBP-1-specific primers that amplify a 297 bp (unspliced) or 271 bp (spliced) fragment. RNA from FRTL5 cells treated with tunicamycin was amplified as control. (**B**) In parallel, cells were lysed in detergent and the indicated proteins were detected by immunoblotting. As a positive control, cells were treated with 10 μg/ml tunicamycin for 8 hours to induce CHOP expression, and for 3 hours to induce XBP-1 splicing and eIF2α phosphorylation. Treatments were started at 3 hours after activation.

Discussion

Thyrocytes respond to TSH stimulation by increasing Tg synthesis and secretion. Because Tg is a challenging ER client (Di Jeso et al., 2003; Kim and Arvan, 1991; Kim et al., 1992), optimal folding conditions are needed to maintain secretion. We found that by an early boost of synthesis of ER-resident chaperones and folding enzymes, stimulated thyrocytes prevented unfolded protein accumulation. This resulted in remodeling of the thyrocytes and their ER without an ER lumenal stress signal.

The anticipation of thyrocytes to the upcoming change in protein secretion was obvious from differential changes in their protein synthesis rates. Upon stimulation, metabolic activity of the thyrocytes changed: whereas synthesis of household proteins such as actin remained constant, synthesis rates of other clusters of proteins changed dramatically. ER-resident protein synthesis peaked early, and at these times Tg synthesis was repressed relative to synthesis of other proteins. For at least 24 hours, synthesis rates of ER-resident proteins remained higher than before activation. Eventually their synthesis levels decreased again, perhaps because the required increase in folding capacity had been reached. This is consistent with an important characteristic of thyrocytes: they respond to cycles of increasing and decreasing demand for Tg production, and they are long-lived cells. This means that thyrocytes

survive these changes: unlike the antibody-secreting plasma cells thyrocytes do not die after a few days of massive protein secretion. These cells find a new equilibrium instead and continue to function. Because XBP-1 splicing was undetectable at all times during stimulation, we concluded that the remodeling of thyrocytes follows a signal transduction cascade induced by the TSH incubation, which may involve UPR components but avoids and prevents an ER lumenal unfolded protein stress.

The difference in expression kinetics and steady-state changes of the various resident ER proteins may cause stoichiometries to change in subtle ways in the ER, changing ER function slightly. It is reasonable to assume that proteins required by Tg for its folding would take precedence. This cannot be determined from known data, however, as Tg folds in a large protein complex (Kim and Arvan, 1991), and most of the known and abundant ER proteins were found to associate with Tg during its maturation (Kim and Arvan, 1991; Kim et al., 1992; Sargsyan et al., 2002; Di Jeso et al., 2003; Di Jeso et al., 2005). Moreover, considering the incomplete annotation of sequenced genomes, Tg is likely to recruit other, as yet unidentified, folding assistants in the ER. Only an increase in information on the ER residents and Tg folding will answer this.

Avoiding protein stress has obvious advantages for a specialized secretory cell. Activation of the UPR usually occurs by sensing of compromised folding capacity and accumulation of unfolded protein (reviewed by Ron and Walter, 2007), in other words, when the situation in the ER is escalating. Under normal conditions, ER chaperones and folding enzymes reach millimolar concentrations, greatly outnumbering their client proteins (Marquardt et al., 1993; Stevens and Argon, 1999). The ability to maintain this productive chaperone to client ratio lessens the burden on the cell, precludes investment of energy in refolding misfolded proteins, and allows faster adaptation. Specifically, activation of PERK would attenuate protein synthesis precisely when increased protein production is essential. In B-lymphocytes, where ATF6 is activated at later stages of differentiation (Gass et al., 2002) and XBP-1 is essential for full expansion of the ER (Tirosh et al., 2005), PERK is indeed not activated (Gass et al., 2008). For the reversibly changing thyrocytes, the situation is more complex. eIF2α is phosphorylated in the resting state in the non-activated thyrocytes. This must be catalyzed by kinases other than PERK, for lack of an ER lumenal stress signal (Ron, 2002). eIF2α is known to be part of the integrated stress response, where ER stress and broad cellular stresses meet (Ron, 2002). Although hormone addition augmented eIF2α phosphorylation for a short time, the eIF2α-kinases were silenced while Tg expression increased, confirming our XBP-1 results that ER stress is never sensed in these cells. Still, our findings show that they are highly capable of mounting an ER stress response when triggered with a UPR-inducing agent. In summary, there is no ER stress in the thyrocytes, either before or after activation by hormone.

Differentiating B-lymphocytes are precedent for upregulation of ER folding capacity in the absence of an ER lumenal stress. The initial phase of ER expansion happens before synthesis of antibody subunits is massively upregulated and before XBP-1 is activated (Romijn et al., 2005; van Anken et al., 2003). The ER expands even in the absence of XBP-1 (Reimold et al., 2001; Tirosh et al., 2005), and XBP-1 splicing does not require antibody synthesis (Hu et al., 2009). In the B cell the initial increase of folding capacity is regulated by a developmental program rather than by accumulation of unfolded protein in the ER, which is in line with a finale of programmed cell death. Although thyrocytes undergo regular ER expansion and shrinkage without consequence for viability, their strategy in essence is the same. Avoiding ER stress is not limited to secretory cells, as Brewer and colleagues (Brewer et al., 1997) showed that a panel of non-professional secretory cells, after depletion of essential growth factors, responded to re-addition of the growth factor by upregulating BiP and GRP94 mRNA before a general increase in glycoprotein synthesis. CHOP, which is a secondary UPR messenger implicated in promoting apoptosis (Marciniak et al., 2004), was not induced in the thyrocytes, implying absence of strong or persistent ER stress (Rutkowski et al., 2006). ER expansion as a preventive measure to avoid stress signals appears to be a general strategy of cells.

If unfolded protein stress is not the signal for ER expansion, then what is? TSH activates a G-protein-coupled receptor on thyrocytes (Szkudlinski et al., 2002), which activates G proteins associated with the internal loops of the receptor, leading to production of multiple signaling molecules that can activate a broad variety of signaling pathways and transcription factors (Kimura et al., 2001). Potential targets are transcription factors of the cAMP response element binding (CREB) protein family. The CREB transcription factor family consists of basic region-leucine zipper (bZip) proteins that bind to the same consensus-activating site, termed an ATF/CRE element (reviewed by Hai and Hartman, 2001). ATF4 and ATF6, two members of this transcription factor family, induce expression of ER-resident chaperones either via binding to ER stress elements and UPR responsive elements (Kokame et al., 2001; Wang et al., 2000; Yoshida et al., 1998; Yoshida et al., 2003) or via binding to non-stress-related promoter sites (Ma and Hendershot, 2004). Use of UPR transcription factors for this regulation implies a physiological response without the unfolded protein stress. ATF4 and ATF6 have been reported to be activated in the TSH-stimulated FRTL-5 cells and have been suggested to be at least partly responsible for the induction of ER chaperones (Sargsyan et al., 2004). The signaling pathway that was initiated by binding of TSH to its receptor activates both Tg and a number of transcription factors that induce expression of ER-resident proteins. Not only does the activation of the Tg gene require binding of CREB to its atypical CRE element, but translation of Tg is controlled as well, by Pax-8, TTF-1 (Kambe et al., 1996) and CAF (Berg et al., 1997), which allows an extra level of control over the correct timing of Tg protein production.

Which transcription factors activate the genes of ER-resident proteins under non-stressed conditions is one of the next questions to be answered. A candidate is CREB3 (or Luman), which can bind to the UPR responsive element, but is not activated by classical UPR stimulants such as tunicamycin or thapsigargin (DenBoer et al., 2005). But are there others? And are the classical UPR transcription factors required? Do the transcription factors that specialized secretory cells use also maintain steady-state chaperone levels? Which as yet unidentified promoter elements are present in the genes of ER-resident chaperones and folding enzymes? Up to now, the promoter regions of the ER-resident proteins that we identified in this study have not been characterized completely. Most contain a range of UPR and ERS elements, next to other regulatory sites and many unknowns. The expression clusters we found do not correlate with any known regulatory cluster or component, which is probably because of this dearth of knowledge. Future studies will certainly provide answers to our questions.

In conclusion, we have shown that in activated thyrocytes the production of the major ER client protein Tg was delayed while ER folding capacity expanded. This emerges as a general strategy

for various types of cells: thyrocytes that constantly adapt their ER content to hormonal stimuli, B lymphocytes that follow a well-orchestrated developmental program ending in apoptosis, and non-secretory cells adapting to growth stimuli. The stress signal from unfolded or misfolded cargo activates a valuable back-up system, but under physiological conditions the increasing load on the organelle is met by pre-emptive adaptation of the levels of ER folding machinery to prevent the stress.

Materials and Methods

Cell culture and activation

The FRTL5 cell line was grown in Coon's modified Ham's F-12 medium (Euroclone) supplemented with 5% FBS (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), and a mixture of hormones and growth factors (6H), consisting of 1 mU/ml TSH, 5 μg/ml transferrin, 10 ng/ml somatostatin, 10 ng/ml glycyl-1-histidyl-1-lysine, 10 nM hydrocortisone (all from Sigma) and 10 μg/ml insulin (Invitrogen). Cells were kept in a humidified incubator at 37°C in 5% (v/v) CO_2. Before activation, at a confluency of 50%, cells were kept in medium supplemented only with 0.5% FBS, penicillin and streptomycin for 5 days. To activate the cells, the medium was replaced with normal culture medium.

Metabolic labeling

At the indicated time points after activation cells were starved in MEM without cysteine and methionine (ICN Biomedicals) for 15 minutes and labeled for 30 minutes using 100 μCi Redivue Promix ^{35}S cell labeling mix (GE Healthcare Biosciences) per 10-cm dish. Before lysis, cells were washed twice with ice-cold HBSS containing 20 mM NEM. Lysis buffer consisted of 25 mM Hepes-KOH pH 7.0, 0.25% sucrose, 3% Triton X-100, 10 μg/ml each of the protease inhibitors chymostatin, leupeptin, antipain, pepstatin, and 1 mM PMSF (phenylmethylsulphonyl fluoride).

SDS-PAGE and western blot analysis

The protein concentration of lysates was determined using a Lowry assay (Bio-Rad). Samples were normalized to the total amount of protein, and heated to 95°C for 5 minutes in Laemmli sample buffer containing 50 mM DTT (dithiothreitol) for reduction. Samples were loaded onto 6% and 10% SDS-PA gels for denaturing gel electrophoresis (Laemmli, 1970). Kodak Biomax MR film or storage phosphor screens (Kodak) were exposed to the gels. Signals were detected with the Bio-Rad Personal Molecular Imager FX and non-saturated exposures were quantified using the ImageQuant software package (Bio-Rad).

Alternatively, proteins were blotted onto nitrocellulose membranes (Schleicher and Schuell). Membranes were saturated using 3% Protifar (Nutricia) in PBS containing 0.1% Tween 20. BiP and PDI were detected using polyclonal rabbit sera (Benham et al., 2000), and thyroglobulin was recognized by polyclonal rabbit serum M0781 (DAKO; used for all experiments that identified Tg), or polyclonal goat serum sc-7836 (Santa Cruz Biotechnology; supplementary material Fig. S1). CHOP was detected by a monoclonal mouse serum MA1-250 (Affinity Bioreagents), eIF2α (total) was detected by mouse monoclonal 3D5 (Sigma), phosphorylated eIF2α was detected by rabbit polyclonal E2125 (Sigma), and actin was detected by A2066 (Sigma). After incubation with HRP-labeled secondary antibody (Bio-Rad) a chemiluminescence reaction was performed using homemade ECL solutions (Leong and Fox, 1990).

Two-dimensional gel electrophoresis

Gel electrophoresis was performed essentially as described before (van Anken et al., 2003). Briefly, 18 cm IPG (immobilized pH gradient) strips (pH 3-10 NL, GE Healthcare Biosciences) were rehydrated overnight with approximately 200 μg protein dissolved in 7.7 M urea, 2.2 M thiourea, 3% Triton X-100, 15 mM DTT, 0.5% IPG carrier ampholytes, and Bromophenol blue. Focusing was performed for at least 10 hours at a final voltage of 3500 V. After equilibration in 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 65 mM DTT, proteins were alkylated and separated on 10% or, when indicated, 12.5% SDS-PA gels. Proteins were visualized using silver staining, gels were scanned using a Bio-Rad GS710 scanner, and scans were analyzed and quantified using the PDQuest software package (Bio-Rad). Spots were excised from the gel for identification using mass spectrometry or gels were dried and Kodak Biomax MR films or storage phosphor screens (Bio-Rad) were exposed to the gels. Signals from radiolabeled proteins were detected with the Bio-Rad Personal Molecular Imager FX and non-saturated exposures quantified using the PDQuest software package (Bio-Rad).

Mass spectrometric analysis of protein spots

Spots were excised from the 2D gels, and peptide mixtures from in-gel tryptic digests (Roche Diagnostics, GmbH, sequencing grade) were concentrated. Matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF-MS) analysis was performed on an Applied Biosystems 4700 ToF-ToF mass spectrometer (Framingham, MA) in reflectron mode using a *m/z* 700–5000 Da mass range. Data were acquired at a 200 Hz laser repetition rate, a 20 kV accelerating voltage, a 70% grid voltage and a digitizer bin size of 1.0 nanoseconds. Peptide collision-induced (CID) MS/MS, was performed at a collision energy of 1 kV with an indicated collision gas pressure of ~1.10^{-6} Torr.

Protein identification

Data analysis was carried out using GPS explorer Software V3.5 (Applied Biosystems) with integrated Mascot search (Matrix Science, UK). Searches were against the NCBI database. Further settings: trypsin with two missed cleavages, carbamidomethyl (C) as fixed modification, oxidation (M), N-acetylation (N-terminus) and phosphorylation (of S, T and Y) as variable modifications. Peptide tolerance was set to 100 p.p.m. for MS and 0.2 Da for MS/MS tolerance. A threshold for confident protein identification was set at a protein Mascot score >60 and more than two peptides per protein with a minimal peptide score of 25. In all cases the confidence index (C.I. parameter) in the identification was 100%.

Clustering analysis

Mev4.0 software was used for an unbiased clustering analysis (Saeed et al., 2003). The self-organizing tree algorithm (SOTA) (Dopazo and Carazo, 1997; Herrero et al., 2001) constructs a binary tree (dendrogram) in which the terminal nodes are the resulting clusters. The program goes through a preset number of iterative cycles in which every time the most diverse protein set is split into two clusters, based only on similarity of expression profile. The first step splits the total protein set into two, the next splits the most diverse set, generating three clusters, and so on. The program continues until the number of preset clusters (number of cycles plus 1) has been reached, or when a cluster falls below the threshold of maximal diversity allowed within a cluster. The parameters we used for our analysis were 10 cycles and a maximum cell diversity of 0.9 (Dopazo and Carazo, 1997; Herrero et al., 2001). Hence this is a completely unbiased clustering mechanism that does not take any other parameter than expression pattern into account.

RNA isolation, reverse transcription and PCR

Cells were lysed in Trizol (Invitrogen) and total RNA was isolated according to the manufacturer's instructions. Reverse transcription was performed using the First Strand cDNA Synthesis kit (Fermentas) according to supplied instructions. XBP-1-specific primers were used to amplify 297 bp (unspliced) or 271 bp (spliced) fragments. PCR products were visualized on 2.4% agarose gels and stained with ethidium bromide. Forward primer: 5′-GAAGAGAACCACAAACTCCA-3′; reverse primer: 5′-GGATATCAGACTCAGAATCT-3′.

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