Quality control of glycosylphosphatidylinositol anchor attachment in mammalian cells: a biochemical study

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hGHDAF28 is a chimaeric protein consisting of human growth hormone fused to a crippled signal sequence for glycosylphosphatidylinositol (GPI)-anchor addition from decay-accelerating factor, and serves as a model for quality control of GPI-anchor addition. hGHDAF28 is retained in a pre-Golgi compartment and degraded intracellularly by a mechanism with similarity to that for other endoplasmic reticulum (ER)-retained proteins (Field, Moran, Lee, Keller and Caras (1994) J. Biol. Chem. **269**, 10830–10837). We have studied the specific pathway of degradation for hGHDAF28 using a number of compounds which affect protein folding and trafficking pathways in eukaryotic cells. We found that high concentrations of dithiothreitol (DTT) accelerated loss of hGHDAF28 by degradation from cell lysates, without promoting secretion or alteration of disulphide-bond distribution, in contrast to a number of other examples of ER-retained proteins where DTT alters disulphide-bond formation. Additionally, degradation of hGHDAF28 was sensitive to pH, being promoted at pH 6.0 and inhibited at pH 8.0; however, the latter effect was transient, indicating incomplete blockade. Degradation was also partially enhanced by depletion of ER calcium with thapsigargin, but this was again a partial and transient effect. Furthermore, degradation was temperature sensitive, with a gradual decrease in rate observed at lower temperatures. However, a sharp decrease in turnover between 15 °C and 20 °C, indicative of a requirement for transport to a post-ER compartment, was not observed. Degradation of hGHDAF28 was insensitive to treatment with nocodozole or compounds preventing cytoplasmic autophagy, suggesting that ER degradation is independent of classical autophagy and microtubule-dependent processes. In addition, disruption of N-glycosylation with tunicamycin, or inhibition of processing of immature N-glycan chains with castanospermine or deoxynojirimycin, had little effect on the stability of hGHDAF28, suggesting that disruption of the BiP/calnexin quality-control system by bulk cellular secretory proteins does not influence the ER-degradation pathway of hGHDAF28. Intermolecular hGHDAF28 cysteine bonds result in the formation of aggregates which are probably important in the retention of the molecule. The insensitivity of this structure to reduction *in vivo*, together with the enhanced degradation rate, indicates that DTT mediates its effect on stability via a molecule involved in degradation of hGHDAF28, possibly a thiol-sensitive protease.

INTRODUCTION

Mechanisms by which eukaryotic cells ensure that the products of biosynthesis are accurate are of extreme importance, and for secretory proteins such control is frequently mediated by selective intracellular retention and degradation [1]. One of the earliest events is retention within the endoplasmic reticulum (ER); in these cases the retained protein is denied access to the Golgi apparatus and is degraded, but the precise location of the degradative site remains an unresolved issue [2]. We are using a protein with an unprocessable glycosylphosphatidylinositol (GPI) signal sequence as a model to study this event [3,4] and its importance in the quality control of GPI-addition.

GPI-anchor processing proceeds via addition of a preformed glycolipid to a site close to the C-terminus of a polypeptide following translocation across the ER membrane (see [5] for recent review). The GPI-signal is a tripartite structure containing a hydrophobic sequence at the C-terminus, a three-amino-acid cleavage site and a hydrophilic spacer between them [5]. The loss of GPI processing results in failure of the protein to reach the cell surface and its degradation. The C-terminal signal sequence is normally cleaved from the protein during anchor addition, but its continued presence acts as a determinant for retention, thus providing a quality control mechanism for GPI-addition. Truncation of the hydrophilic spacer can partially alleviate the block to ER egress [4], consistent with the GPI signal sequence being a bona fide ER-retention signal. A recent report indicates that this type of mechanism is conserved in *Saccharomyces cerevisiae*, and that failure to add a GPI-anchor excludes the misprocessed protein from ER transport vesicles [6].

hGHDAF28 contains the entire human growth hormone (hGH) sequence fused to a non-functional GPI signal derived from decay-accelerating factor (DAF), and is retained in a pre-Golgi compartment and colocalizes with ER–Golgi intermediate compartment 53 (ERGIC-53) at the light level [3]. It is also present in numerous cytoplasmic vesicles distinct from the ER, a subset of which also contains cathepsin D [4], a marker for lysosomes and mature autophagosomes. By electron microscopic analysis, hGHDAF28 is detected in dense vesicles containing complex membrane whorls with similarity to intermediate autophagosomes and degrading hydroxymethylglutarate-Co A reductase (HMG-R)-induced karmellae [7–10], suggesting a possible relationship between ER-retention and autophagy. It was suggested that the degradation of ER-derived material was mediated by a process distinct from autophagy [10]. Additionally, degradation of components of the Sec61p complex in *S. cerevisiae*

Abbreviations used: ASGR, asialoglycoprotein receptor; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CST, castanospermine; DAF, decay-accelerating factor; DNJ, deoxynojirimycin; DTT, dithiothreitol; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment; FCS, fetal calf serum; GPI, glycosylphosphatidylinositol; hGH, human growth hormone; HMG-R, hydroxymethylglutarate-CoA reductase; NP40, Nonidet P40; PI-PLC, phosphatidylinositol-specific phospholipase C; HRP, horseradish peroxidase; TBS, Tris-buffered saline; TCA, trichloroacetic acid; TX-100, Triton X-100; VSV, vesicular stomatitis virus.

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is achieved via the ubiquitin-proteosome pathway [11], and therefore ER-degradation may proceed via a number of distinct pathways. Indeed, Oda et al. [12] recently obtained evidence to suggest involvement of the proteosome in GPI quality control.

Biochemical criteria indicate that hGHDAF28 is degraded in a non-lysosomal compartment by a mechanism similar to that described for the T-cell receptor [2]: degradation is prevented by low temperature and ATP depletion, suggestive of a sequestration/transport step from the site of polypeptide synthesis. Considerable amounts of disulphide-linked homoaggregates of hGHDAF28 are formed, with the possible interpretation that, in common with other amphipathic molecules, hGHDAF28 forms micellar structures with the GPI-signal domains forming a hydrophobic core [4].

In studies, *in vivo*, of the early steps in folding of other ER-retained proteins, use has been made of reagents that affect intra-ER redox and calcium homeostasis [13,14]. Dithiothreitol (DTT) is a relatively hydrophobic molecule which can cross cellular membranes and reduce the normally oxidizing environment of the ER. Disulphide-bond formation and rearrangement are often crucial for the stability of folded proteins [15], although it is apparent that perturbation of redox or calcium concentration affects only a subset of ER-degraded proteins [16], indicating the existence of a number of distinct routes for disposal of ER proteins. We have studied the requirements for degradation of hGHDAF28 using a panel of compounds acting on protein processing, transport, autophagy and cytoskeletal function, and assessed the role of pH and temperature, in order to clarify the relationship between ER retention and degradation and to understand in greater detail quality control of GPI-anchor addition.

MATERIALS AND METHODS

Nucleic acids and transfections

The hGHDAF28 minigene insert (a 2.4 kb *Eco*RI fragment) and recombinant hGH were a gift from Dr. Ingrid Caras (Department of Neurobiology, Genentech Inc., San Francisco, CA, U.S.A.). The eukaryotic expression vector, pRK5, containing a synthetic horseradish peroxidase (HRP) gene was the gift of Dr. Dan Cutler (University College, London, London, U.K.). The HRP gene, cloned into *Bam*HI sites, was removed, the vector religated and hGHDAF28 inserted into the *Eco*RI site within the polylinker. The hGHDAF29 expression construct was produced by PCR-mediated mutagenesis of the hGHDAF28 insert (M. C. Field and D. Nesbeth, unpublished work), introducing a serine residue at the GPI-attachment site (see [4] for a description of the constructs) following movement of the hGHDAF28 insert into the plasmid pSG5 (Stratagene Inc.). The mutated region was correct by sequence analysis, and hGHDAF29 was expressed on the cell surface and released by *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C digestion (PI-PLC) (D. Nesbeth and M.C. Field, unpublished work), confirming the presence of the GPI anchor. Plasmid DNA was prepared from large volume cultures in *Escherichia coli* XL1-Blue cells using a Qiagen kit. COS 7 or Hep2 cells were cultured in Dulbecco's modified Eagle's medium containing 10 % (v/v) heat-inactivated fetal calf serum (FCS) plus L-glutamine, 5 units/ml penicillin and 5 μg/ml streptomycin (all from Sigma) in a 5 % CO_2/95 % air atmosphere at 37 °C.

On the day preceding transfection, cells were subcultured into 35-mm dishes. Typically, 1 ml of cell suspension was added to each dish to ensure equal plating density. On the day of transfection cells were inspected microscopically to check uniformity of plating density and the health of the cultures. Cells were transfected using Lipofectamine™ (Gibco-BRL), essentially following the manufacturer's instructions. Transfection reagent (6 μl) with 1 μg of plasmid in Optimem (Gibco-BRL) was used per 35-mm dish of cells, and incubated for 5 h. The transfection reagent was made up fresh each time, but in a batch sufficient for all cultures to be transfected to ensure that equal amounts of DNA and Lipofectamine were delivered to each dish. Cells were allowed to recover by the addition of 1 vol. of complete medium, containing 20 % (v/v) FCS, for at least 16 h before study.

Metabolic labelling and immunoprecipitation

Cells were metabolically labelled by starving in methionine-free minimal essential medium (Gibco-BRL) containing 10 % (v/v) dialysed FCS for at least 1 h before addition of label. [^{35}S]Methionine (NEN-DuPont) was added at ~75 μCi/35-mm dish. Cells were chased by the addition of either excess cold methionine (Sigma) in PBS or complete medium. For [^{3}H]proline (Amersham International) labelling experiments, cells were pulse-labelled (200 μCi/ml) in Dulbecco's modified Eagle's medium (which contains no proline) supplemented with 10 % (v/v) dialysed FCS. Cells were detached from the substrate with 50 mM EDTA in PBS (detachment checked microscopically) and lysed with 100 μl of lysis buffer [1 % Nonidet P40 (NP40)/6 mM EDTA in PBS containing 1 μl of protease-inhibitor cocktail (2 mg/ml each of leupeptin, antipain, pepstatin and chymostatin plus 100 μg/ml tosyl-lysylchloromethane added dry)]. The cells were incubated on ice for 15 min, the nuclei spun out in a microfuge and the supernatant boiled following addition of 13 μl of 10 % (v/v) SDS. The lysate was then diluted to 1 ml with dilution buffer {1.25 % Triton X-100 (TX-100) in Tris-buffered saline [TBS, 50 mM Tris/HCl (pH 7.5)/150 mM NaCl] plus 6 mM EDTA} and precleared with 50 μl of pansorbin suspension (Calbiochem) at 37 °C for 60 min, before transfer to a fresh tube and collection of the target antigen with rabbit antibodies against hGH or HRP and 25μl of Protein A–Sepharose. Typically, 1 μl of antiserum was used per 35-mm dish of cells kept overnight at 4 °C with gentle rocking. Immunocomplexes were pelleted, washed twice with buffer [0.1 % (v/v) TX-100/0.2 % (v/v) SDS/5 mM EDTA in TBS], once with a high-salt buffer [0.02 % TX-100/50 mM Tris/HCl (pH 7.5)/1 M NaCl] and once with PBS, before final resuspension in 50 μl of 2 × SDS sample buffer. The immunocomplexes were dissociated and solubilized by immediate heating to 98 °C for 5 min, and then either analysed immediately or stored at −20 °C. For reduced samples, DTT was added fresh from a 1 M stock to a final concentration of 50 mM, and the sample was heated at 98 °C for 5 min before electrophoresis. In all kinetics experiments a control time course was performed to ensure that any variability between experiments could be normalized. Within experiment duplicates routinely gave < 5 % variance, whilst between experiment variance was $\leqslant 10$ %. For studies at decreased temperature, medium pre-equilibrated at the appropriate temperature was added at the end of the pulse and the cells were chased as normal. Where pH alterations were studied, medium containing carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at 0.5 μM, and adjusted to the required pH, was added at the start of the chase.

Antibodies and enzymes

Polyclonal antisera to recombinant hGH were raised in rabbits or mice using RIBI™ as adjuvant (Sigma). Antibody production was monitored by indirect immunofluorescence. Affinity purification of rabbit anti-hGH antibodies was performed using

40 % ammonium sulphate-fractionated immunized rabbit serum on CNBr-Sepharose (Pharmacia-LKB)-immobilized recombinant hGH (Genentech Inc.), following standard procedures. Antiserum to HRP was from SeroTech. Fluorescein- and rhodamine-conjugated anti-rabbit or anti-mouse antibodies, fluorescein isothiocyanate-phalloidin and anti-tubulin antibodies were from Sigma. Antibody to ERGIC-53 was a gift from Dr. Hans-Peter Hauri (Basel University, Basel, Switzerland). N-glycanase, endoglycosidase H and *B. thuringiensis* PI-PLC were from Boehringer Mannheim. N-glycanase digestions were performed in 50 mM ammonium bicarbonate, pH 8.0, and PI-PLC and endo H digestions in PBS, typically for 2 h at 37 °C. Control digests omitted the enzyme.

Compounds and treatments

Cleland's reagent (DTT), vinblastine, nocodozole, taxol, CCCP, sodium azide, sodium fluoride, thapsigargin, cyclohexamide, leucine, asparagine, Nonidet P40 (NP40), TX-100, Protein A–Sepharose, leupeptin, chymostatin, antipain, pepstatin and tosyllysylchloromethane were all from Sigma. Castanospermine (CST), deoxynojirimycin (DNJ), and tunicamycin were from Calbiochem. Compounds for addition to living cells were made up in PBS, tissue-culture grade DMSO (Sigma) or ethanol, at a concentration such that the final volume added did not exceed 20 μl/ml. Cells were pretreated, or the compound was added with the chase medium, as indicated.

A number of the compounds were tested to ensure that each batch was active and produced the expected biological effect. We used the glycosylation of HRP expressed in COS cells as an assay for tunicamycin, CST and DNJ. Untreated HRP migrates on SDS/PAGE as a 52 kDa protein, but N-glycanase digestion reduced this to $\sim$ 38 kDa, identical with the migration of HRP expressed in tunicamycin-treated cells (results not shown). CST and DNJ treatment resulted in decreased SDS/PAGE mobility (apparent molecular masses of 58 and 54 kDa respectively), consistent with the retention of α-glucose residues and abolition of oligomannose glycan trimming (results not shown). Digestion with endoglycosidase H increased the migration of CST, DNJ and control-cell-expressed HRP to the $\sim$ 38 kDa position, confirming the altered molecular masses as being due to carbohydrate processing. We used immunofluorescence to assess the cytoskeletal agents. Both nocodozole and vinblastine caused ERGIC-53 to alter location from a perinuclear position, typical of the ERGIC, to smaller, numerous, dispersed vesicles in the cytoplasm, confirming ERGIC disruption, a previously observed effect of nocodozole. In addition, we assessed the ability of the cells to export the HRP glycoprotein. As exocytosis does not require microtubules, this parameter was used to ensure that any effects on hGHDAF28 were not due to toxicity. For vinblastine, taxol and nocodozole, HRP export was found to be $>$ 75–100 % of control levels, indicating no substantial detrimental effect.

Immunofluorescence

Cells for immunofluorescence were grown on glass coverslips, transfected and treated as usual. Following pharmacological manipulations, cells were washed and fixed for 10 min with 3.7 % formaldehyde, then permeabilized with 0.5 % (v/v) TX-100 for 12 min (both in PBS) at room temperature. The cells were then blocked with 10 % FCS in PBS for at least 30 min, incubated with primary antibody followed by secondary antibody and mounted using Vectashield (Vector Labs). Slides were inspected using an NFX35 fluorescence microscope (Nikon).

Trichloroacetic acid (TCA) precipitation

To determine total protein synthesis, triplicate COS cell cultures were labelled for 1 h with [^{35}S]methionine as above, harvested and resuspended in 100 μl of SDS/PAGE sample buffer. Lysates were separated in 15 % (v/v) reducing SDS gels, and the total radioactivity incorporated into macromolecular material was quantified by PhosphorImager (Molecular Dynamics, Inc.) analysis. Alternatively, cells were labelled for 1 h with [^{35}S]-methionine as above, harvested and precipitated with 55 μl of ice cold 100 % TCA. The suspension was filtered through a 2.5 cm glass microfibre filter (Whatman GF/C) and washed thoroughly with 10 % TCA followed by 100 % ethanol. Discs were transferred to scintillation liquid and total incorporation was measured on a scintillation counter. Determinations were done in triplicate.

Gel electrophoresis

Immunoprecipitates were analysed in 12 or 15 % SDS/polyacrylamide gels by addition of 50 μl of SDS-containing sample buffer directly to the Protein A beads, followed by heating to 98 °C as described above. Samples, typically 15 μl, were loaded using a Hamilton syringe. After electrophoresis through minigels at 15 V/cm (constant voltage), the gels were fixed with methanol/acetic acid/water (2:1:7, by vol.), impregnated with En^{3}Hance (NEN Dupont) and dried. The gels were then exposed to AR-XOMat film (Kodak) at $-$85 °C or a PhosphorImager screen (Molecular Dynamics, Inc.). Bands were quantified using a PhosphorImager system or by scanning the autoradiograph using a Microtek Scanmaker 2 with Adobe Photoshop software (Adobe Systems Inc.), and quantified using NIH Image (NIH).

RESULTS

DTT alters the recovery of hGHDAF28 from COS cells

hGHDAF28 is recovered from cells in monomeric (25 kDa), dimeric (52 kDa) and higher-order aggregates, which migrate predominantly at the interface between the stacking and resolving portions of a discontinuous SDS/polyacrylamide gel. The native hGH molecule contains two disulphide bonds, whilst the DAF tail introduces an unpaired cysteine into the C-terminal region. Upon reduction, essentially all hGHDAF28 migrates as the reduced-hGHDAF28 monomer (29 kDa), indicating that the hGHDAF28 aggregate, as isolated, contains essentially only that polypeptide (see [4] for detailed discussion). Alteration in migration of the reduced versus unreduced monomer, due to opening of intrachain disulphide bonds, and distribution of monomer, dimer and aggregate were used to monitor the effect of DTT on disulphide bonding within retained hGHDAF28. We initially examined the recovery of the transgene product across a four orders of magnitude DTT concentration range. DTT (1 μM–10 mM) was present during a 60 min [^{35}S]methionine labelling period, the cells were then lysed in the presence of 20 mM *N*-ethylmaleimide to trap folding intermediates and prevent oxidation. High concentrations of DTT ($\sim$ 1 mM–10 mM) resulted in a decreased yield of hGHDAF28 (Figure 1A). When these data were quantified we found that the yield of hGHDAF28 was decreased by $\sim$ 45 % by 5 mM DTT (Figure 1B). We also observed that low concentrations of DTT (0.1 mM) increased the yield of hGHDAF28 by $\sim$ 10 %. When these immunoprecipitates were examined under non-reducing conditions, the distribution of the molecular forms of hGHDAF28 was unaltered (Figure 1C), and therefore the effect on recovery was not mediated by alteration in the disulphide-

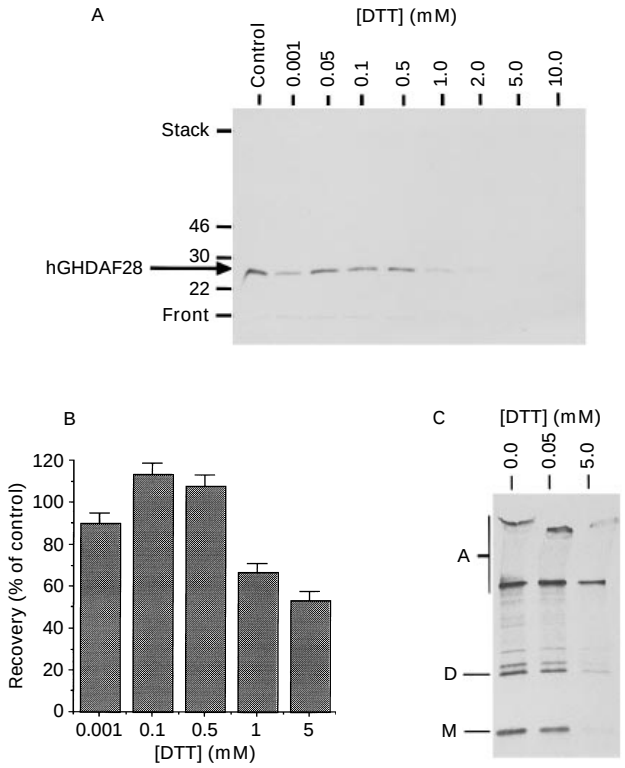

Figure 1 DTT treatment *in vivo* affects recovery of hGHDAF28

(**A**) Autoradiograph of reduced SDS/PAGE analysis of hGHDAF28 immunoprecipitates from cells pulse-labelled for 1 h with [^{35}S]methionine treated with various concentrations of DTT as indicated. The positions of molecular mass markers in kDa and of the stacking gel interface (Stack) and dye front (Front) are shown. Note that essentially all of the radiolabelled macromolecular material is recovered as the hGHDAF28 band. (**B**) Quantification of the hGHDAF28 band by PhosphorImager from two experiments similar to (**A**). Recovery of hGHDAF28 in untreated cells is set at 100%. DTT concentrations are indicated in mM. Data are means ± S.E.M. (**C**) Unreduced SDS/PAGE analysis of hGHDAF28 immunoprecipitates from cells treated with 0, 50 μM and 5 mM DTT and pulse-labelled for 1 h with [^{35}S]methionine. Positions of monomer, dimer and aggregates are indicated as M, D and A respectively. A large number of bands are visualized in this experiment compared with the single band obtained on reduction (**A**).

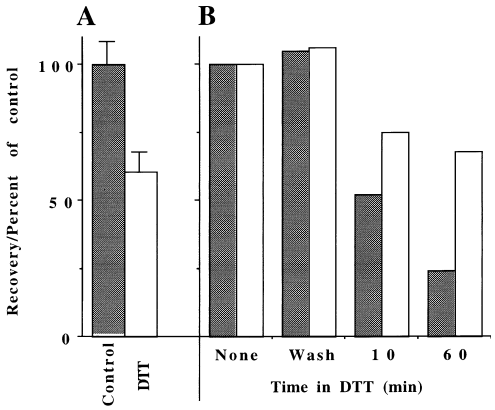

Figure 2 Incorporation of [^{35}S]methionine into hGHDAF28 is highly sensitive to DTT

(**A**) DTT decreases the incorporation of [^{35}S]methionine into total cell protein. Cells were pulse-labelled in the presence (open bar) or absence (black bar) of 5 mM DTT for 1 h and total protein was separated on 15% SDS gels and radioactivity quantified by PhosphorImager. Data are the means of three separate experiments and error bars indicate S.D. (**B**) Yields of hGHDAF28 and ssHRP recovered by immunoprecipitation from cells labelled for 60 min with [^{35}S]methionine and treated with 5 mM DTT for 60 min (60), 10 min (10) or during the wash only (see text). Black bars indicate hGHDAF28, open bars HRP.

bond distribution. To determine whether hGHDAF28 is sensitive, we examined hGHDAF28 treated with DTT and pulse-labelled for only 5 min. Again the reduced 29 kDa form was not observed on non-reducing gels (results not shown), suggesting that hGHDAF28 is either completely insensitive to DTT or rapidly enters a DTT-resistant state following synthesis.

Effect of DTT on secretory pathway proteins

We next chose to determine whether the effect of DTT was similar for other proteins imported into the ER lumen. The soluble secretory protein HRP was selected as, like hGHDAF28, it is imported into the ER in a signal-sequence-dependent manner, but unlike hGHDAF28, it is not membrane associated, is highly glycosylated, contains several disulphide bonds and is secreted. Therefore, any effect of DTT on ER delivery would be the same, but the subsequent folding and export pathways would be expected to be different. COS 7 cells expressing signal-sequence HRP or hGHDAF28 were pulse-labelled for 60 min, and DTT was added at the beginning of the pulse, during the last 10 min, or in the cell wash buffer only, and signal-sequence HRP and hGHDAF28 were recovered by immunoprecipitation. Recovery of hGHDAF28 was far more sensitive to DTT than signal-sequence HRP; a 60 min treatment resulted in less than 25% recovery of hGHDAF28 compared with nearly 70% recovery of HRP, compared with the control (Figure 2B). The fact that folded HRP is stable to DTT treatment is in agreement with this observation [17]. The sensitivity of hGHDAF28 was also apparent after a 10 min treatment with DTT, and therefore is rapid in onset. DTT was also found to affect similarly incorporation of [^{35}S]methionine into hGHDAF29 when pulse-labelled for 60 min, but did not affect GPI processing (D. Nesbeth and M. C. Field, unpublished work), confirming the generality of the phenomenon and also ruling out a specific role for the GPI signal sequence in hGHDAF28 DTT sensitivity. The additional sensitivity of hGHDAF28 compared with HRP suggested that DTT may be exerting other effects, specific to metabolism of the transgene product (see below).

Total protein synthesis is decreased at high DTT concentrations

To determine whether DTT affected total protein synthesis we analysed total cellular protein incorporation of [^{35}S]methionine by SDS/PAGE (Figure 2A). A 40% reduction in total protein synthesis was apparent in the presence of 5 mM DTT, similar to the effect on hGHDAF28. We also analysed total protein synthesis by TCA precipitation; again approx. 50% reduction in total protein synthesis was apparent in the presence of 5 mM DTT [2.7×10^6 compared with 5.3×10^6 c.p.m. in controls ($n = 3$)]. In addition, total protein was labelled with [^{3}H]proline and the effect of DTT on incorporation into protein was assessed by SDS/PAGE followed by PhosphorImager analysis. A dramatic decrease, of almost 90%, in labelling of polypeptides was observed when DTT was present (20.5 ± 2.75 PhosphorImager units against 2.45 ± 0.65 units; values ± S.E.M., $n = 2$). Taken together these data indicate that DTT has a general effect in decreasing protein synthesis. The effect is rapid in onset, as brief exposure to the reagent results in a clear decrease in synthesis (Figure 2B).

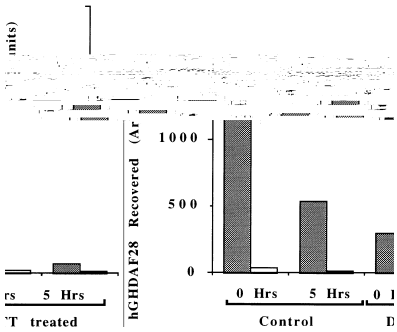

Figure 3 Decreased yield of hGHDAF28 in DTT-treated cells is not due to insolubility

COS cells expressing hGHDAF28 were metabolically labelled for 1 h, and either lysed immediately or chased for 5 h before lysis with NP40 lysis buffer (see Materials and methods section). The insoluble pellets were then re-extracted with lysis buffer supplemented with 1% SDS. Both extracts were independently subjected to the immunoprecipitation procedure for quantification of hGHDAF28. Black bars, recovery with 1% NP40; open bars, additional recovery with 1% SDS. Radioactivity in hGHDAF28 is expressed as PhosphorImager counts.

Extraction of grossly misfolded hGHDAF28

We considered that decreased yield of hGHDAF28 was possibly due to inability to extract the protein. Addition of DTT to cells results in an accumulation of reduced, misfolded proteins within the ER [14], therefore gross aggregation of hGHDAF28 may account for a decrease in recovery with 1% NP40. We sequentially extracted pulse-labelled cells expressing hGHDAF28, first with 1% NP40 and then with 1% SDS, and then quantified recovery by immunoprecipitation and SDS/PAGE. No decrease in the NP40-extractable fraction of hGHDAF28 was found if the cells were pretreated with DTT (Figure 3). Similar results were obtained if we re-extracted with 5 M urea instead of 1% SDS (not shown). Therefore reduced recovery was not simply due to NP40 insolubility.

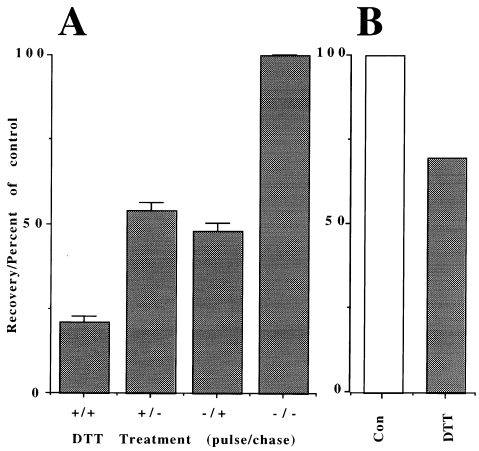

Figure 4 DTT has both translational and post-translational effects on hGHDAF28

(**A**) Bar graph showing recovery of hGHDAF28 from cells pulse-labelled and chased for 1 h and subjected to different conditions during pulse and chase. Treatments are designated as pulse conditions before slash, chase after. '+' indicates cells were incubated with 5 mM DTT, '−' indicates no addition. Data are the means of three experiments and error bars indicate S.D. (**B**) Cells were pulse-labelled for 5 min in the presence or absence (Con) of DTT, and hGHDAF28 was quantified by immunoprecipitation and SDS/PAGE analysis.

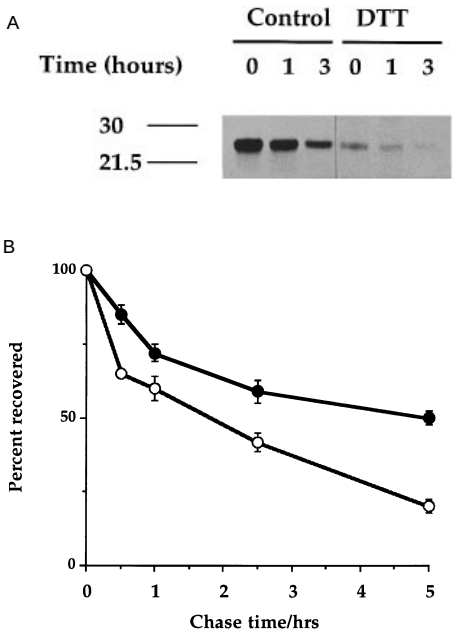

Figure 5 High concentrations of DTT decrease the half-life of hGHDAF28

Cells expressing hGHDAF28 were pulse-labelled for 1 h and chased for the indicated times. The amount of hGHDAF28 remaining was quantified by immunoprecipitation and SDS/PAGE analysis under reducing conditions, followed by visualization by PhosphorImager or autoradiography. (**A**) Autoradiogram showing 15% reducing SDS/PAGE analysis of hGHDAF28, pulse-labelled and chased in the presence or absence of 5 mM DTT. Note the overall decrease in intensity of the bands in the DTT-treated samples. (**B**) Quantification of the degradation of hGHDAF28 in the presence and absence of DTT. Control (●) and 5 mM DTT-treated (○) cells. Data are the means of three separate experiments with S.D.s indicated.

Translational and post-translational effects of DTT

We next considered whether DTT alters hGHDAF28 stability. Transfectedcells were labelled for 1 h, either in the presence (+) or the absence (−) of 5 mM DTT. Post-translational effects of DTT on hGHDAF28 were analysed by adding excess methionine and chasing, again in the presence (+) or absence (−) of DTT for an additional 1 h. When DTT was present during labelling (+/− versus −/−), recovery was reduced by ~ 50%. However, removal of DTT after peptide synthesis increased the yield of hGHDAF28 (+/− versus +/+), indicative of post-translational effects (Figure 4A). Similar results were obtained where the pulse length was decreased to 5 min, eliminating the possibility of rapid degradation during the pulse being responsible for the decrease in yield (Figure 4B).

DTT accelerates degradation of hGHDAF28

The results above suggest that DTT decreases the stability of hGHDAF28. In order to examine the kinetics of hGHDAF28 turnover directly in DTT-treated cells, transfected COS 7 cells were pulse-labelled and chased with and without DTT. Overall, the half-life of hGHDAF28 was approximately halved in the presence of 5 mM DTT (Figure 5). No significant difference in

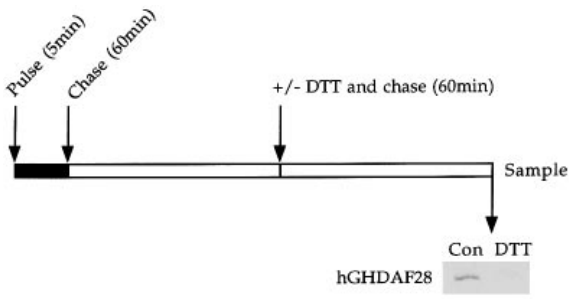

Figure 6 DTT effect is long-lived

Cells were pulse-labelled for 5 min, chased for 60 min, and 5 mM DTT was added for a further 60 min. HGHDAF28 was then recovered by immunoprecipitation. Left-hand lane, control cells; right-hand lane, DTT-treated cells.

the rate of degradation was observed between control and 100 μM DTT-treated cells (results not shown). Analysis of these immunoprecipitates on non-reducing gels and PhosphorImager quantification demonstrated that all forms of hGHDAF28, i.e. aggregate and monomer, are degraded with similar kinetics, ruling out the possibility that one form matures into another before ultimate disposal (results not shown). hGHDAF28 was not recovered from the medium and therefore was not being aberrantly secreted (results not shown).

hGHDAF28 degradation retains sensitivity to DTT after an extended period

We next investigated whether hGHDAF28 degradation would lose DTT sensitivity over time. Transfected COS 7 cells were pulse-labelled for 5 min and then chased for 60 min to allow potential egress of proteins from the ER into the ERGIC. DTT (5 mM) was then added and the cells were chased for a further 1 h before lysis and recovery of hGHDAF28 (Figure 6). We observed that degradation of hGHDAF28 was still enhanced by DTT treatment.

Perturbation of calcium levels

The ER is a major intracellular reservoir of calcium, and the high lumenal concentrations of Ca^{2+} may be essential for correct folding of proteins, interaction with chaperones and even retention of ER-resident proteins [18–20]. Therefore, release of Ca^{2+} from the lumen of the ER may have a significant effect on the folding and retention of hGHDAF28, as seen with other ER-retained proteins [13]. However, not all ER-retained proteins are degraded by Ca^{2+}-sensitive processes [16]. Depletion of calcium from the ER by treatment with thapsigargin, a specific inhibitor of the ER Ca^{2+}-ATPase, resulted in a partial, transient enhancement of degradation at 1 h of chase (Figure 7). However, by 3 h the effect was negligible and completely lost at 6 h. The location of hGHDAF28 in thapsigargin-treated cells was also unaffected when analysed by immunofluorescence (results not shown). This observation is comparable to the H1 and H2 subunits of the asialoglycoprotein receptor (ASGR), where lowering of ER Ca^{2+} enhanced misfolding and degradation [13].

Degradation of hGHDAF28 is temperature- and pH-dependent

Degradation of hGHDAF28 is not affected by lysosomotrophic reagents such as ammonium chloride or chloroquine, which serve to raise lysosomal pH [4]. However, the effect of altering pH

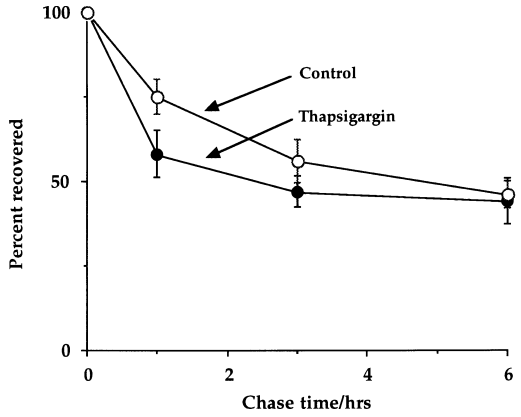

Figure 7 Calcium depletion augments degradation of hGHDAF28

Cells expressing hGHDAF28 were pulse-labelled and chased for the indicated times in the presence or absence of thapsigargin. The amount of hGHDAF28 remaining was quantified by immunoprecipitation and SDS/PAGE analysis under reducing conditions, followed by visualization by PhosphorImager. Degradation curves for hGHDAF28 immunoprecipitated from control and 100 nM thapsigargin-treated cells are indicated by (○) and (●) respectively. Data are the means of four separate experiments; S.D.s are indicated. Note that treating cells with 500 nM thapsigargin in an additional experiment produced essentially identical results with 100 nM thapsigargin.

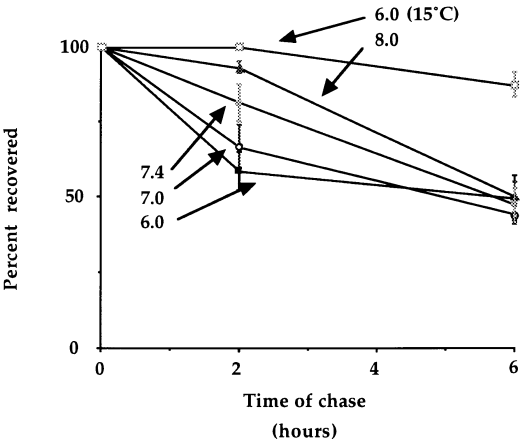

Figure 8 Degradation of hGHDAF28 is enhanced at low pH and inhibited at high pH

Cells expressing hGHDAF28 were pulse-labelled for 1 h at pH 7.4 and pH clamped (at the indicated pH values) by addition of 0.5 μM CCCP at the start of the chase. Recovery was assessed by immunoprecipitation and resolution of 15% reducing SDS/polyacrylamide gels. Results shown are the means of two experiments with the S.E.M. indicated.

using a CCCP pH clamp has been reported for T-cell receptor subunits where raised pH inhibited degradation [2], and we therefore re-examined the effect of pH on hGHDAF28 stability. The results indicate that the degradation rate was enhanced by decreased pH (Figure 8). Similarly, lowering temperature to 15 °C results in a dramatic decrease in degradation of hGHDAF28 and other ER-retained proteins, and this has been taken as evidence for a transport step being important in the degradation process [2,4]. We re-examined the effect of temperature and observed that, for hGHDAF28, the rate of degradation varied continuously with temperature (Figure 9); i.e. a sharp break in the rate was not apparent in the 15–20 °C range (15 °C inhibits ER exit in mammalian cells).

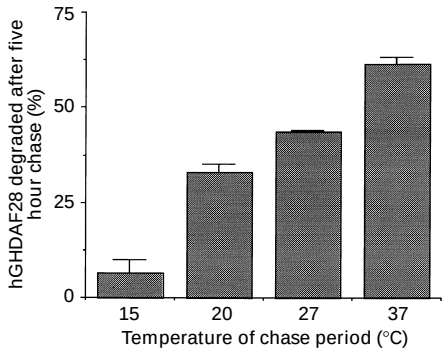

Figure 9 Degradation of hGHDAF28 is continuously variable with temperature

Cells expressing hGHDAF28 were pulse-labelled for 30 min at 37 °C and chased for the indicated times at 37, 27, 20 and 15 °C. hGHDAF28 was quantified by immunoprecipitation and resolution on 15% reducing SDS/polyacrylamide gels. Data shown are the means of three experiments with the S.D. shown (except at 20 °C, where $n = 2$).

Table 1 Effects of various compounds on the stability of hGHDAF28 in transiently transfected COS cells

Cells were pulse-labelled for 1 h following the procedures detailed in the Materials and methods section, and sampled 1 h after the addition of chase medium. The number of experiments (n) is indicated. The degradation index is expressed as $[Counts_{t=0\,h} - Counts_{t=1\,h}]^{Treated}/[Counts_{t=0\,h} - Counts_{t=1\,h}]^{Control}$ by PhosphorImager quantification following immunoprecipitation and resolution of hGHDAF28 on 15% reducing SDS/polyacrylamide gels. A number smaller than 1.0 indicates inhibition; the value of 0.27 for NaN_3, a positive control, represents almost complete protection of the hGHDAF28 band (> 95% recovered after a 3 h chase period). *Compounds were batch tested for activity, as described in the Materials and methods section. †Sodium azide used in the presence of 20 mM sodium fluoride. ‡The same result was obtained for a similar experiment performed using a stable CHO cell transfectant (M. C. Field and I. Caras, unpublished work). §Index measured at 0.5 h chase.

Drug	Pretreatment (h)	Degradation index (% error)	n
10 μg/ml Nocodozole*	4.0	0.95 (7.9)	3
10 μM Taxol*	4.0	0.88 (19.5)	2
0.1% NaN_3†	1.0	0.27 (4.2)	2
25 mM Asparagine	1.0	1.22 (11.1)	2
5 mM 3-Methyladenine‡	2.0	1.26	1
1 μM Vinblastine	2.0	0.90	1
10 μM Vinblastine§	2.0	1.22 (6.2)	2
1 mM Castanospermine*	2.0	0.80 (3.1)	3
1 mM Deoxynojirimycin*	2.0	1.13 (6.9)	3
10 μg/ml Tunicamycin*	2.0	0.96 (8.1)	3
20 μg/ml Cyclohexamide	2.0	1.1	1

Autophagy

We next chose to address the relationship between ER-retention/degradation and autophagy. The endocytotic-derived path is sensitive to 3-methyladenine, whilst the ER-derived arm is inhibited by asparagine [21,22]. Also the entire pathway is inhibited by vinblastine, a microtubule disrupting agent. 3-Methyladenine had no effect on degradation of hGHDAF28 (Table 1). This finding is consistent with the known action of this compound, which is believed to affect the endosome-derived autophagic path [21]. As hGHDAF28 never reaches the Golgi complex it is unlikely that these elements are involved. Similarly, asparagine had no effect on hGHDAF28 stability, suggesting that ER-type autophagy is not involved in hGHDAF28 turnover. Finally vinblastine, a more general inhibitor of autophagy, had no effect (Table 1). These results suggest that the degradation of hGHDAF28 is distinct from cytoplasmic-type autophagic mechanisms.

Glycosylation and folding

Tunicamycin, which prevents N-glycan addition, increases protein misfolding in the ER, whilst inhibition of trimming of α-glucose residues from the $Glc_3Man_9GlcNAc_2$ species with CST and DNJ prevents interaction of proteins with calnexin [23,24]. We reasoned that, as these compounds have major effects on protein folding in the ER, they may influence hGHDAF28 turnover by increasing general ER degradation [25]. Tunicamycin and DNJ had no significant affect on hGHDAF28 stability, whilst CST slightly inhibited degradation; together these results argue that hGHDAF28 is unaffected by the calnexin retention system. We also investigated whether protein synthesis was required for degradation of hGHDAF28: we treated cells with cyclohexamide immediately following pulse-labelling, but this also had no effect on degradation of hGHDAF28 (Table 1).

Cytoskeletal elements

Earlier work has suggested that hGHDAF28 may be transported via the ERGIC, or some other compartment, to cathepsin D-containing cytoplasmic vesicles, presumably distinct from lysosomes [4]. We proposed previously [4] that these structures were autophagic vesicles, but the evidence described above indicates that autophagy is not likely to be involved in disposal of hGHDAF28. In the present study we have attempted to gain some insight into the nature of possible post-ER compartments involved in hGHDAF28 turnover by disruption of microtubule function with nocodozole and taxol. We found that both compounds had no significant effect (Table 1), suggesting that intact functional microtubules are unimportant in hGHDAF28 degradation, which is in agreement with our findings with vinblastine.

DISCUSSION

In this study we have used a panel of reagents and manipulations with well-characterized effects on trafficking and protein processing to gain insight into the requirements for retention and degradation of misprocessed GPI-anchored proteins. We have investigated redox, Ca^{2+}, pH, temperature, glycosylation, cytoskeletal function and autophagy. Under stress conditions, fragments of ER membrane, together with lumenal contents, are pinched off and mature into lysosome-like structures [7,8,21] in a process termed autophagy. Visualization of electron-dense structures near to the ER resembling autophagosomes containing hGHDAF28, and partial colocalization of hGHDAF28 and cathepsin D in cytoplasmic structures [4], implicated autophagic mechanisms in hGHDAF28 turnover. These structures are broadly similar to Russell bodies [26], also potentially involved in ER-degradation processes, but the precise relationship between the ER and these compartments is not known. To determine whether a classical cytoplasmic autophagy pathway is involved in disposal of hGHDAF28 we used several compounds which shut down the pathway [21,22]. As we were unable to inhibit the degradation, our results indicate that hGHDAF28 degradation is independent of classical autophagy. There are precedents for

such pathways: examples include, degradation of HMG-R-induced karmellae in yeast [10] and higher eukaryotes [16], disposal of yeast Sec61p [11] and turnover of a truncated ribophorin variant [27]. Interestingly, degradation of HMG-R and a variant of α_1-antitrypsin are both sensitive to blockade of protein synthesis [28,29], which is not the case for hGHDAF28. For Sec61p and the CFTRΔF508 mutant there is strong evidence that ubiquitination and proteosome activity are primarily responsible for removal of cytoplasmic domains; the significance of this mechanism to lumenal disposal is not known [30]. However, similar mechanisms may also be involved in the present system, as the proteosome has recently been implicated as having a role in GPI-quality control, although the precise role that proteosome function may play in ER-related degradation events has yet to be defined [12]. Overall, there are much data to indicate the presence of several pathways for ER degradation; however, the determinant(s) responsible for directing a given protein into a specific route remain unknown at the present time.

We did not observe significant inhibition of hGHDAF28 degradation using nocodozole, which depolymerizes microtubules. ER-retained proteins can recycle between the ER and ERGIC, but only retrograde transport is microtubule dependent [31], suggesting that the cycling of ERGIC contents is unimportant for degradation. Therefore the simplest interpretation is that degradation only takes place in the ER. Immunofluorescence implies the build up of hGHDAF28 at an ER-exit site when transport is blocked by cold temperatures, suggesting that a proportion of hGHDAF28 may recycle, as seen for the ts045 vesicular stomatitis virus (VSV) G-protein at a non-permissive temperature [32] and for unassembled MHC Class I molecules [33]. Interestingly, a subpopulation of cells ($> 10\,\%$) contain hGHDAF28 in a nocodozole-sensitive compartment, which appears similar to the staining obtained with p53 under the same conditions (M. C. Field, unpublished work), suggesting that, in some cells at least, a small proportion of hGHDAF28 may be located within the ERGIC. It is also possible that the ERGIC provides an additional checkpoint for retention, so that any material that has escaped the ER is retained by the ERGIC and recycled back to the ER for disposal. This small amount may be insignificant in bulk terms and so not detectable by our assay, and is clearly of minor importance in the present context.

Experimental perturbation of protein folding can result in increased heavy chain binding protein expression, an important component of the quality control process. We considered that interference with quality control functions by increasing the level of unfolded proteins in the ER with tunicamycin, i.e. decreasing the concentration of free heavy chain binding protein, and by eliminating the $Glc_1Man_9GlcNAc_2$ calnexin ligand, observed in other systems to increase ER degradation [25], may affect hGHDAF28 stability through induction of quality control proteins. We found that inhibition of α-glucosidases I and II, preventing recognition of glycoproteins by calnexin [23], had little effect. CST produced a small transient increase in hGHDAF28 stability, but DNJ, with a similar site of action, did not elicit a similar response and therefore this is not likely to be significant. Similarly, inhibiting all N-glycan addition with tunicamycin did not alter the hGHDAF28 half-life. Previously we showed that hGHDAF28 aggregates do not contain other proteins, even when misfolding is enhanced by tunicamycin [4]. These results suggest that hGHDAF28 is not influenced by the general folding and quality control mechanisms that involve the heavy chain binding protein and/or calnexin, and lead us to propose that the formation of aggregates is most likely a rapid self-assembly process that does not require lumenal ER proteins.

Calcium has been implicated in a number of ER processes. Depletion can augment degradation of the ASGR H2 subunit, inhibit degradation of truncated ribophorin, cause misfolding of the H1 ASGR subunit and lead to loss of KDEL-mediated retention and escape of otherwise ER-retained proteins, e.g. T-cell receptor α-subunit, whilst other proteins, e.g. the VSV G-protein, are unaffected [15,13,18,27,34]. Calcium effects have been ascribed both to the calnexin retention system, ruled out in the glucosidase inhibitor experiments described above, and a calcium-rich matrix within the ER, but there are many other possible sites of action. Thapsigargin produces a small enhancement of degradation of hGHDAF28, but overall the effect is not major and argues that degradation is not strongly influenced by any Ca^{2+}-requiring process. The small effect may reflect general perturbation of ER conditions rather than any specific target, and is certainly not as large an effect as seen for the ASGR H2 subunit [35].

In common with other ER-retained proteins, we have previously shown that hGHDAF28 degradation is unaffected by the lysosomotrophic reagent ammonium chloride [4], which is considered to act by raising the pH of lysosomal compartments. We directly examined the pH dependence of hGHDAF28 degradation by using the proton ionophore CCCP as a pH clamp. We observed that increasing pH resulted in inhibition of turnover, whilst decreased pH augmented degradation of hGHDAF28. This is essentially identical with the observations for TCR α-chains [2], and implies that a slightly acidic environment is optimal for hGHDAF28 turnover. This could be the optimum pH of a critical enzyme at any point along the degradation pathway and, as for the observations with DTT (below), without additional information cannot be taken as evidence for the conditions within the hGHDAF28 degradative compartment. We also investigated the temperature dependence of hGHDAF28 turnover. This was also found to be similar to the TCR α-chain situation; decreasing temperature resulted in a lower turnover rate, and at 15 °C 95 % of the hGHDAF28 was protected for an extended period. Whilst this behaviour has been interpreted as evidence for a transport step to a post-ER compartment [2], the possibility that the observations reflect thermal dependence of a critical enzyme in the degradative pathway cannot be ruled out by these results alone. An Arrhenius plot of our data also revealed a discontinuity between 15 and 20 °C (results not shown), as noted by Lippincott-Schwartz et al. [2]. Therefore degradation of hGHDAF28 and of the TCR α-chain is similar for two important parameters, pH and temperature. As the C-terminus of the TCR chain is highly similar to a GPI-signal sequence (a C-terminal hydrophobic sequence with no hydrophilic C-terminal cytoplasmic domain), and both of these moieties are ER-retention motifs, it is not surprising that both proteins behave in a similar fashion, and therefore most probably enter the same degradation pathway. Conversely, HMG-R and the TCR α-chain are degraded by distinct mechanisms when co-expressed in the same cell [36].

We studied the effect of altering cell redox conditions in some detail. Native hGHDAF28 contains two disulphide bonds and a single unpaired cysteine; therefore the presence of trimeric and higher order multimers indicates non-native disulphide bonding in hGHDAF28 in addition to the native monomer [4]. DTT does cause ER-retention of certain otherwise secreted proteins, presumably due to alteration in the redox equilibrium of the normally oxidizing ER, resulting in misfolding and recognition by the quality control system [14,37,38]. We considered that DTT may have one or more effects, including enhanced misfolding, induction of quality control proteins due to an increased concentration of folding proteins in the ER, and possible inhibition of association with the ER matrix, mediated by disulphide bonds

[39]. Our data clearly demonstrate that hGHDAF28 degradation is stimulated by 5 mM DTT without alteration of the distribution of molecular forms. Therefore unfolding of hGHDAF28 and a simple increase in protease or chaperone accessibility cannot be the mechanism of stimulation. This is consistent with the insensitivity to tunicamycin and glucosidase inhibition of the process. We can exclude inhibition of protein synthesis, extraction difficulties or loss of material to the medium as accounting for the rapid loss of hGHDAF28.

Our results are therefore consistent with enhanced degradation being mediated via a molecule distinct from hGHDAF28, i.e. a DTT-sensitive factor. This factor could be a thiol protease, as implicated in intracellular degradation of other ER-retained proteins (T-cell receptor subunits and μ-chains [40]). Consistent with this, we have recently obtained direct evidence for the role of a thiol protease in hGHDAF28 degradation, but the identity of this enzyme remains unknown (L. J. Wainwright and M. C. Field, unpublished work). However, it is also possible that the effect of DTT is manifested through a more distal process than direct proteolysis of hGHDAF28, for example processing of a critical ER component, and therefore the DTT-sensitive factor and hGHDAF28-protease may not be the same molecule.

hGHDAF28 retained sensitivity to DTT for some time following synthesis, suggesting either that the protein was retained within the ER, or that similar processes are involved in all compartments to which hGHDAF28 has access. DTT is unable to perturb ERGIC events, as DTT affects VSV G-protein in the ER, but not following transport to the ERGIC [41]. Therefore these results argue for an ER location for hGHDAF28 degradation, also consistent with our observation that disruption of microtubules has no effect on hGHDAF28. Interestingly, our observations are at variance with a recent study on λ-light chains, where 2-mercaptoethanol was used to perturb ER redox [39]. In that study, addition of the reducing agent resulted in secretion of the light chain, whilst retained light chains were found to be associated with a large number of other proteins. The authors proposed that a free cysteine in the retained protein acted as an anchor by disulphide interaction with resident ER proteins. For hGHDAF28 it is probable that the protein aggregates in the absence of interaction with other proteins, and this rapid malfolding leads to retention. Rapidity would also diminish the opportunity for chaperone action. The ability to form large homoaggregates by aberrant disulphide-bond formation most probably explains the difference between hGHDAF28 and the λ-light chain [39].

Whilst investigating the effect of DTT on hGHDAF28 stability, we observed that 5 mM DTT decreased [^{35}S]methionine incorporation into protein, in addition to enhancing the degradation. We saw a similar effect for hGHDAF29, not an ER-retained protein, and to a lesser extent for HRP. A yield reduction was seen recently in studies of gp80 in MDCK cells using DTT, but the mechanism was not investigated by these workers [37]. However, it is highly likely that the phenomenon reported here was also involved in that study. Whilst we do not know the precise mechanism by which DTT can alter incorporation of [^{35}S]methionine into a polypeptide, two possibilities are uptake from the medium and the efficiency of polypeptide-chain elongation. The former is unlikely as we also saw severe impairment of polypeptide incorporation of [^{3}H]proline. We can rule out specific promoter effects (hGHDAF28 and hGHDAF29 are expressed from different plasmid systems), which is consistent with the general decrease in [^{35}S]methionine incorporation seen by SDS/PAGE analysis of total proteins, rather than loss of specific polypeptides. Clearly these observations have an important bearing on the use of DTT, and suggest that protein synthesis levels must be monitored in any investigation where the compound is used *in vivo*.

A pathway for the intracellular disposal of misprocessed GPI-anchored proteins is now known to be present in mammals and *Dictostelium discoidium* [4,42] and is probably as widespread as the GPI-anchor itself. Our observations suggest rather a robust and intrinsic ER-proteolysis pathway for the disposal of these proteins, which does not interact with other polypeptides that are still undergoing folding reactions.

In this report we have studied degradation of hGHDAF28, a probe for GPI quality control, using a number of well-characterized compounds and conditions. Our major findings suggest that hGHDAF28 is retained and degraded within the ER itself. We cannot at present rule out that a minor proportion is capable of recycling through the ERGIC. There is no evidence for involvement of other quality control mechanisms, specifically the BiP and calnexin pathways, and retention appears to be largely due to aggregation and is therefore intrinsic to the hGHDAF28 molecule. This is consistent with recent data from studies of *Saccharomyces cerevisiae*, which indicated that the GPI-anchored protein, gas1p, must obtain an anchor for entry into COPII ER-transit vesicles [6] and progression to the *cis*-Golgi. Interestingly, these authors did not observe rapid degradation of the unprocessed gas1p, suggesting that in GPI, quality control may be divergent between yeasts and mammals, although presumably misprocessed proteins must be degraded in some fashion by the yeast cell, and indeed an ER-degradation system analogous to the mammalian process is present. The nature or existence of the ERGIC in yeast is at present not firmly established, and this may have some bearing on differences in quality control processes between *S. cerevisiae* and higher eukaryotes.

We have found that a number of factors distinguish the hGHDAF28 degradation pathway from that of other ER-retained proteins. For example, the VSV G-protein is misfolded by DTT treatment [32], the ASGR H2 subunit is cleaved in the absence of ATP, and some proteins are degraded more slowly following calcium depletion. None of these applies to hGHDAF28. However, in pH- and temperature-dependence hGHDAF28 is most similar to the TCR α-chain pathway. The C-terminus of this protein is very similar in structure to the GPI addition signal of hGHDAF28, and the similarity in turnover may simply reflect the physicochemical properties of the two proteins. An alternative possibility is that there exists a specialized mechanism for recognition of this class of protein; most recently the involvement of the ubiquitin/proteosome pathway in HMG-R turnover and possibly GPI quality control suggests that highly sophisticated mechanisms for recognition of unwanted ER components do indeed exist. Clearly a number of different GPI-anchored proteins will need to be studied in order to determine if there is a GPI-specific pathway.

Formation of the hGHDAF28 aggregate probably occurs very rapidly, with no opportunity for ER chaperones to interact before irreversible misfolding occurs. Indeed, aggregate formation is insensitive to DTT within 5 min of synthesis, and the aggregates do not contain other proteins, as detected by co-immunoprecipitation [4]. These aggregates are resistant to DTT, as are fully folded forms of influenza haemagglutinin and HRP [17,43]. We have recently detected several different forms of hGHDAF28 by partial digestion of aggregates with proteases *in vitro*, suggesting some order within the aggregates (L. J. Wainwright and M. C. Field, unpublished work). We intend to exploit glycosylated and GPI signal variants of hGHDAF28 to investigate these and other possibilities more directly in future work.

We thank Dr. Ingrid Caras, Genentech Inc., for the hGHDAF28 insert and recombinant hGH, Dr. Dan Cutler, University College London, for the ssHRP.pRK5 plasmid, Dr. H. P. Hauri, Basel University, for the p53 antibody, and Mr. D. Nesbeth, Imperial College London, for the hGHDAF29 construct. This work was supported by a project grant from the Medical Research Council of Great Britain to M.C.F.

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Received 19 July 1996/16 September 1996; accepted 23 September 1996