The EMBO Journal vol.12 no.12 pp.4755–4761, 1993

Quality control of ER synthesized proteins: an exposed thiol group as a three-way switch mediating assembly, retention and degradation

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Communicated by R.Sitia

Plasma cells secrete IgM only in the polymeric form: the C-terminal cysteine of the μ heavy chain (Cys575) is responsible for both intracellular retention and assembly of IgM subunits. Polymerization is not quantitative, and part of IgM is degraded intracellularly. Neither chloroquine nor brefeldin A (BFA) inhibits degradation, suggesting that this process occurs in a pre-Golgi compartment. Degradation of IgM assembly intermediates requires Cys575: the monomeric IgMala575 mutant is stable also when endoplasmic reticulum (ER) to Golgi transport is blocked by BFA. Addition of the 20 C-terminal residues of μ to the lysosomal protease cathepsin D is sufficient to induce pre-Golgi retention and degradation of the chimeric protein: the small amounts of molecules which exit from the ER are mostly covalent dimers. By contrast, when retained by the KDEL sequence, cathepsin D is stable in the ER, indicating that retention is not sufficient to cause degradation. Replacing the C-terminal cysteine with serine restores transport through the Golgi. As all chimeric cathepsin D constructs display comparable protease activity *in vitro*, their different fates are not determined by gross alterations in folding. Thus, also out of its normal context, the μ chain Cys575 plays a crucial role in quality control, mediating assembly, retention and degradation.

Key words: cathepsin D/degradation/endoplasmic reticulum/immunoglobulin/secretion

Introduction

As they proceed along the exocytic pathway, newly synthesized membrane and secretory proteins undergo several sequential post-translational modifications. The fact that only properly folded and assembled molecules complete their journey through the cell implies the existence of quality control events linked to discrete transport steps (Hurtley and Helenius, 1989; Klausner, 1989). In the case of the T cell receptor (TCR), at least two mechanisms have been shown to divert structurally immature molecules from the bulk-flow: retention in the endoplasmic reticulum (ER) and targeting to lysosomes (Bonifacino and Lippincott-Schwartz, 1991 and references therein). While the latter is a dead end pathway, ER retention not only prevents transport of folding or assembly intermediates, but probably keeps them in an environment optimal for their structural maturation (Helenius *et al.*, 1992). Due to mutations, unbalanced subunit synthesis or inefficient folding and assembly, not all membrane and secretory proteins succeed to attain the proper conformation. Selective degradation is thus required for maintaining a homeostatic equilibrium in the ER. Evidence has been obtained that a proteolytic system, independent from that of lysosomes, is active in a pre-Golgi compartment, possibly the ER itself (for reviews see Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991). In the absence of definitive information about its biochemistry and subcellular localization, pre-Golgi degradation is generally defined by (i) its insensitivity to lysosomotropic drugs, such as NH_4Cl or chloroquine, (ii) the accumulation of the degradation substrate in the ER and (iii) the lack of maturation of the substrate oligosaccharide moieties, characteristic of ER-retained proteins (Lippincott-Schwartz *et al.*, 1988). Furthermore, degradation is generally not inhibited by drugs which perturb transport at various steps along the exocytic pathway. Amongst these, the fungal antibiotic brefeldin A (BFA) disrupts the equilibrium between the anterograde and the retrograde ER–Golgi transport pathways. As a result, protein secretion is blocked, and some of the Golgi markers are relocalized into the ER (Misumi *et al.*, 1986; Lippincott-Schwartz *et al.*, 1990). In general, despite the architecture of the exocytic pathway is severely disturbed, pre-Golgi degradation is not inhibited by BFA (reviewed by Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991).

A key question underlying the process of pre-Golgi degradation is what determines its selectivity. Studies on TCR biosynthesis suggest that the presence of charged residues within the hydrophobic transmembrane region is sufficient to determine rapid degradation of unassembled subunits (Bonifacino *et al.* 1990a,b, 1991; Wileman *et al.*, 1990). As the same residues are involved in subunit assembly (Manolios *et al.*, 1990), a competition between oligomerization and degradation in the plane of the membrane might explain the fate of newly synthesized TCR subunits in the ER. Alternatively, the presence of charged residues can impair the function of the TCRα-transmembrane region as a stop–transfer sequence and thereby determine the complete translocation and degradation in the ER lumen (Shin *et al.*, 1993).

In this study, we have investigated the relationships between assembly, retention and degradation for a soluble oligomeric protein, IgM. Obeying the rules of quality control, IgM is secreted by plasma cells only in the polymeric form [pentamers and hexamers (Davis *et al.*, 1989; Sitia *et al.*, 1990; Randall *et al.*, 1992)]. Two elements have been identified in secretory μ (μs) heavy (H) chains which act sequentially to prevent transport of unassembled IgM: the first constant domain (C_H1) and the C-terminal cysteine (Cys575). The former, which is masked by assembly with light (L) chains, retains free H chains in the ER by interacting with BiP (Bole *et al.*, 1986; Hendershot *et al.*, 1987;

Hendershot, 1990). Cys575 is responsible for both covalent polymerization and intracellular retention of IgM 'monomers' by disulfide interchange reactions (Alberini *et al.*, 1990; Sitia *et al.*, 1990). Since polymerization is not quantitative, part of IgM is degraded intracellularly (Sitia *et al.*, 1987, 1990). How is the selectivity of degradation determined? Here we show that retention is not sufficient to cause degradation, and that Cys575 is required to target IgM assembly intermediates to pre-Golgi proteolysis.

Results

Pre-Golgi degradation of IgM assembly intermediates

In the absence of L chains, free μ chains are retained and rapidly degraded intracellularly (N[μ1] transfectants; Sitia *et al.*, 1987). To characterize further the intracellular compartment in which degradation occurs, pulse-labelled N[μ1] cells were chased in the presence or absence of BFA, a drug which inhibits ER−Golgi transport (Misumi *et al.*, 1986). As shown in Figure 1, BFA does not alter the kinetics of μ chain degradation, even when added 30 min before the pulse (BFA pretreatment). These data indicate that degradation of free μ chains takes place in a pre-Golgi compartment. This interpretation is consistent with the state of μ chain oligosaccharide moieties, determined by gel mobility (see Figure 1a) and endo-H sensitivity assays, as well as immunolocalization experiments (Sitia *et al.*, 1987; Valetti *et al.*, 1991; C.Valetti and R.Sitia, unpublished data).

Expression of μ chain in J558L, a λ1 L chain producing myeloma, results in assembly and secretion of polymeric IgM (J[μ1] transfectants). While μ-L assembly is very rapid, polymerization occurs with slow kinetics, and is likely to be the major rate limiting step of intracellular transport (Tartakoff and Vassalli, 1979; Sitia *et al.*, 1987, 1990).

Considering what is recovered both in the cell lysates and in the supernatant, about one-third of μ chains are degraded by J[μ1] cells within 4 h of chase (Figure 2). The lysis conditions used ensure a complete solubilization of intracellular IgM (Valetti *et al.*, 1991). Both BFA and chloroquine, a drug which inhibits lysosomal proteolysis (Gonzales-Noriega *et al.*, 1980), have only marginal effects on the turnover of μs chains, indicating that in myeloma transfectants IgM assembly intermediates are degraded in a pre-Golgi compartment. As determined by non-reducing gels (data not shown), polymers do not excede 30% of total intracellular IgM recovered at the end of the chase, independently of the presence or absence of BFA or chloroquine. μ2L2 monomers and μL hemimers are the predominant forms accumulating (Sitia *et al.*, 1987; Valetti *et al.*, 1991) and are likely to be the substrates of degradation.

Replacement of Cys575 with alanine (J[μala575] transfectants) induces secretion of monomeric IgM (Sitia

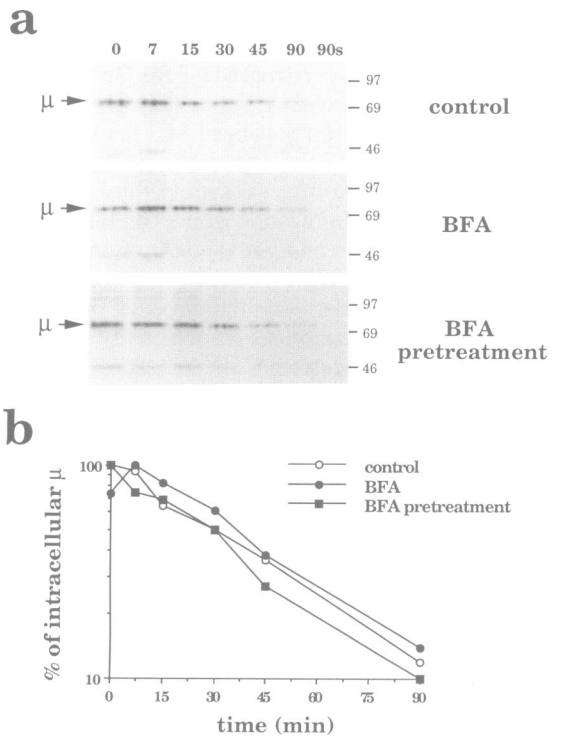

Fig. 1. Pre-Golgi degradation of free μ chains. N[μ1] cells were pulsed for 15 min with [^{35}S]methionine and chased for the indicated times with or without BFA (1 μg/ml) before lysis and immunoprecipitation with anti-μ antibodies. In the lower panel (BFA pretreatment), cells were pretreated for 30 min with BFA before the pulse. Both pulse and chase were performed in the continuous presence of BFA. (**a**) Fluorograms of the H chain region of SDS−polyacrylamide (10%) gels. The last lane on the right (90s) shows the material immunoprecipitated from the supernatants after 90 min of chase. No μ chain is secreted even after prolonged chase periods. (**b**) Relevant bands were quantified by densitometry and expressed as the percentage of the initial μ chain associated radioactiviy.

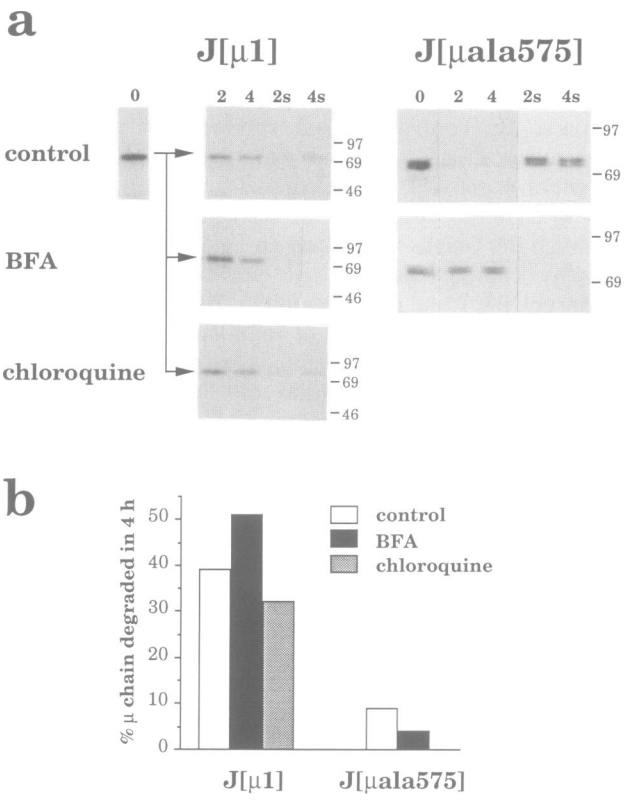

Fig. 2. Pre-Golgi degradation of IgM subunits. (**a**) J[μ1] (left) and J[μala575] (right) were pulsed for 15 min with [^{35}S]methionine and chased for 2 or 4 h as indicated with or without BFA (1 μg/ml) or chloroquine (100 μM). Lysates and supernatants (s) were immunoprecipitated with anti-μ and resolved by SDS−PAGE under reducing conditions (10%). (**b**) The percentage of μ chains degraded after 4 h of chase, calculated by densitometry (μ chain associated radioactivity in the cell lysates + supernatants/initial radioactivity).

et al., 1990) and inhibits intracellular degradation (see right panels of Figure 2a). Replacement of Cys575 with serine yields similar results (not shown). Since in J[μala575] cells both the rate and the efficiency of secretion are increased (Sitia *et al.*, 1990), the faster intracellular transit of IgMala575 might explain the reduction in intracellular degradation. However, blocking the egress from the ER with BFA is not sufficient to induce degradation (Figure 2). Taken together, these results indicate that monomeric IgM is not intrinsically susceptible to proteolysis, and suggest that Cys575 plays a key role in the selective targeting of IgM assembly intermediates to pre-Golgi degradation.

The μs tailpiece is sufficient to transfer retention and degradation to cathepsin D

The plasmid CDM (Pelham, 1988) encodes human cathepsin D tagged with a c-myc epitope (Figure 3). Cathepsin D is an aspartic protease which is routed to lysosomes by mannose-6-phosphate receptors (Kornfeld, 1992). It is synthesized as a ~53 kDa precursor which undergoes proteolytic processing in the endolysosomal compartment to yield a stable (half-life >18 h), covalently linked heterodimer (33+14 kDa) (Hasilik and Neufeld, 1980; Faust *et al.*, 1987; Horst and Hasilik, 1991; Metcalf and Fusek, 1993). While the presence of the c-myc epitope does not alter significantly the intracellular routing of cathepsin D (CDM), addition of the SEKDEL C-terminal hexapeptide (CDMK) induces retention/retrieval in the ER (Pelham, 1988).

To determine whether Cys575 could signal both retention and degradation also out of its normal context, we extended CDM with the 20 C-terminal amino acids of μs (the μ tailpiece, μtp) to generate plasmid CDMμtpCys. In CDM-μtpSer the C-terminal cysteine has been replaced by serine. Figure 3 summarizes the structure of the four constructs utilized in transient expression assays.

During a 2 h chase, part of CDM synthesized in a 15 min pulse is secreted by COS-7 transfectants (Figure 4a, lane 8). The portion of CDM routed to lysosomes is not detectable in cell lysates by immunoprecipitation with 9E10, probably due to cleavage of the c-myc epitope (Pelham, 1988); nevertheless, it can be indirectly estimated by the increase in secretion induced by chloroquine (compare lanes 9 and 8 of Figure 4a) (Gonzales-Noriega *et al.*, 1980).

Fig. 3. Structure of the cathepsin D constructs. Plasmids CDM and CDMK (Pelham, 1988) were kindly given by Dr Hugh Pelham (Cambridge, UK). In CDM, cathepsin D (white broken box) is extended with the c-myc epitope (dark grey box) recognized by the 9E10 antibody. CDMK terminates with the hexapeptide SEKDEL (striped box). The C-terminal extensions of CDMμtpCys and CDMμtpSer (light grey box) are indicated. Note that both contain an N-glycosylation site (NVS) underlined in the figure.

CDMμtpSer is secreted at a level comparable to that of CDM, indicating that further extension at the C-terminus does not alter intracellular transport (lanes 4–6). Saturation of mannose-6-phosphate receptors in COS-7 transfectants might explain why most CDM and CDMμtpSer are secreted rather than transported to the lysosomes (Pelham, 1988; Horst and Hasilik, 1991). The mobility shift in CDMμtpSer is due to the presence of an additional N-glycosylation site in the μtp (underlined in Figure 3). Being conserved from shark to man (Sitia *et al.*, 1990 and references therein) this site might play some important role. A possible function could be that of keeping the relevant cysteine exposed to solvent, thereby affecting retention and degradation. However, the activity of Cys575 in quality control is not affected in a mutant μ chain in which the glycosylation site

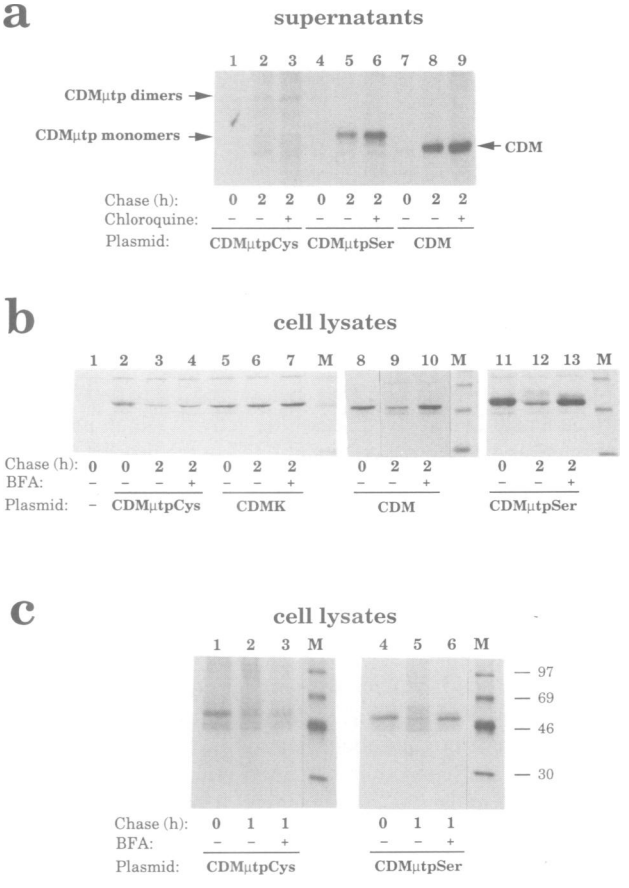

Fig. 4. Pre-Golgi degradation of cathepsin D induced by the μtp. Fourty-eight hours after transfection with the indicated plasmids (CDM, CDMK, CDMμtpCys and CDMμtpSer), COS-7 cells were pulsed for 15 min and chased for the indicated times with or without BFA or chloroquine. Supernatants (panel **a**) and cell lysates (panel **b**) were immunoprecipitated by the monoclonal 9E10 anti-myc antibody and resolved on SDS–PAGE under reducing (panel b, cell lysates) or non-reducing conditions (panel a, supernatants). In panel a, arrows at the left hand margin point to dimeric and monomeric CDMμtp. The three transfectants analysed in panel a synthesized comparable amounts of chimeric cathepsin D (not shown). In panel b, lane M shows the migration pattern of molecular weight markers (bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa). In panel **c**, pulse-labelled CDMμtpCys and CDMμtpSer transfectants were chased for 1 h with or without BFA, and the cell lysates immunoprecipitated with a polyclonal anti-human cathepsin D antibody (a gift of C.Isisoro, University of Torino). Lane M contains molecular weight markers (phosphorylase B, 97 kDa; bovine serum albumin, 69 kDa, ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa).

has been destroyed by replacing Ser565 for Ala (S.Guenzi *et al.*, unpublished data).

In agreement with a crucial role of the μ chain C-terminal cysteine in the mechanism(s) of retention, only traces of CDMμtpCys are secreted (Figure 4a, lanes 1–3). Most of the CDMμtpCys recovered in the supernatant consists of covalent dimers, most probably linked by the tailpiece cysteine (see arrows). In addition, as in the case of IgM (Alberini *et al.*, 1990), the presence of 0.01% 2-mercaptoethanol (2-ME) during the chase period increases secretion of CDMμtpCys (data not shown).

Having established that the C-terminal cysteine is sufficient to cause pre-Golgi retention, we analysed the kinetics of degradation of the chimeric cathepsin D constructs in the presence or absence of BFA. As shown in Figure 4b, ~40% of CDMμtpCys is degraded within 2 h of chase. BFA does not inhibit CDMμtpCys degradation. By contrast, CDMK is stable in the presence or absence of BFA (lanes 5–7). This confirms that retention in the ER is not sufficient to cause degradation. Replacement of the critical cysteine with serine inhibits proteolysis in the presence of BFA, and CDMμtpSer is indeed similar to CDM with respect to intracellular traffic (lanes 8–13).

Degradation of CDMμtpCys is evident also when samples are immunoprecipitated with a polyclonal anti-cathepsin D serum instead of the monoclonal anti-c-myc antibody 9E10 (Figure 4c). Thus, the disappearance of CDMμtpCys cannot be explained simply by cleavage of the c-myc epitope. Also in these conditions, degradation intermediates are not detectable.

When the pH is lowered below 5.5, purified bovine procathepsin D displays proteolytic activity which results in self-cleavage between Leu26 and Ile27 in the propart (Larsen *et al.*, 1993). To determine whether our four chimeric proteins retain such activity, insoluble anti-myc antibodies were used to purify exogenous cathepsin D molecules from the lysates of the different transfectants. When immunoprecipitates are exposed at low pH for 1 h, a smaller form is originated (Figure 5, even lanes, see arrows). Pepstatin A, an inhibitor of aspartic proteases, blocks proteolysis (odd lanes). Since samples contain cysteine and serine protease inhibitors [i.e. phenylmethylsulfonylfluoride (PMSF), aprotinin and iodoacetamide], these data indicate that the proteolytic activity detected is due to the chimeric cathepsin D molecules. The cleaved forms display similar mobility in the four constructs analysed, suggesting that also portions of the C-terminal extensions are removed.

The fact that CDMμtpCys and CDMμtpSer display a comparable proteolytic activity rules out the possibility that retention and degradation of CDMμtpCys are due to gross misfolding, and points to a direct signalling role of the C-terminal cysteine.

Taken together, these results indicate that, when the C-terminal cysteine is present, the μtp can indeed act as a colocalized determinant for oligomerization, retention and degradation in a pre-Golgi compartment. Addition of the μtp can alter the fate of cathepsin D, so as to turn a lysosomal proteolytic enzyme into a substrate of pre-Golgi proteases. Since neither dimerization nor degradation of CDMμtpCys are inhibited by BFA, the three alternative fates of newly synthesized CDMμtpCys are likely to be determined in the same compartment, possibly the ER.

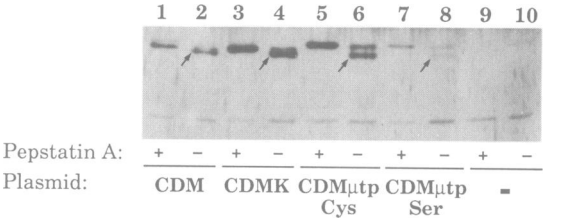

Fig. 5. Retained CDMμtpCys is enzymatically active. Postnuclear supernatants from CDM (lanes 1 and 2), CDMK (lanes 3 and 4), CDMμtpCys (lanes 5 and 6) and CDMμtpSer (lanes 7 and 8) or untransfected COS-7 cells (lanes 9 and 10) lysed in the presence of 20 mM iodoacetamide (an inhibitor of cysteine-proteases) were immunoprecipitated by Sepharose-bound anti-myc (9E10), eluted for 15 min at 37°C in glycine-HCl buffer (pH 3.0) containing 1 mM PMSF and 0.15 U/ml aprotinin A (two inhibitors of serine proteases). Half of the samples contained 1 μM pepstatin (an inhibitor of aspartyl proteases; odd-numbered lanes). Beads were removed by centrifugation and samples were incubated for further 60 min at 37°C before SDS–PAGE. Gels (10%) were blotted to nitrocellulose filters, which were developed by anti-human cathepsin D followed by horseradish peroxidase-conjugated goat anti-rabbit IgG. The main proteolytic products are indicated by arrows.

Slow kinetics of degradation for IgG2b-μtp depend on intrinsic resistance to pre-Golgi proteases

As in the case of cathepsin D constructs, the μtp is sufficient, when attached to γ2b heavy chains, to induce retention of chimeric IgG2bμtp molecules in a pre-Golgi compartment (Alberini *et al.*, 1990; Sitia *et al.*, 1990; Valetti *et al.* unpublished observations). However, the kinetics of degradation of retained IgG2b-μtp are much slower (half-life 10 h) than those of IgM or CDMμtpCys (~2 h). In addition, oligomeric forms of IgG2b-μtp are not detected (Sitia *et al.*, 1990). Also when expressed in the absence of light chains, γ2b chains display a remarkable stability (half-life > 4 h) as compared with μ (<1 h). To compare the susceptibility of μ and γ2b chains with pre-Golgi proteases, we extended the two heavy chains with the transmembrane (TM) and cytoplasmic regions of the TCR α subunit (Bonifacino *et al.*, 1990a,b; Shin *et al.*, 1993). When expressed in J558L, both μTCRαTM and γ2bTCRαTM assemble with L chains and are degraded in a BFA insensitive way (Figure 6). However, the rate of degradation of μTCRαTM is faster (half-life ~ 30 min) than that of γ2bTCRαTM (half-life ~ 60 min). Thus, IgG2b might indeed be intrinsically more resistant to degradation than IgM subunits.

Discussion

Generality of quality control mechanisms

In both lymphoid and non-lymphoid cells, IgM are secreted only in the polymeric form (Cattaneo and Neuberger, 1987; Valetti *et al.*, 1991). Unassembled subunits are retained intracellularly, confirming that the events underlying quality control of newly synthesized proteins are neither tissue nor species specific. Besides being entirely consistent with this generality concept, the results reported above shed some light on the molecular mechanisms involved. Perhaps the crucial observation is that, when Cys575 is present, the μtp is sufficient to induce pre-Golgi retention of cathepsin D, a protease destined to the lysosomal compartment. Several facts argue in favour of a direct role of Cys575 in the

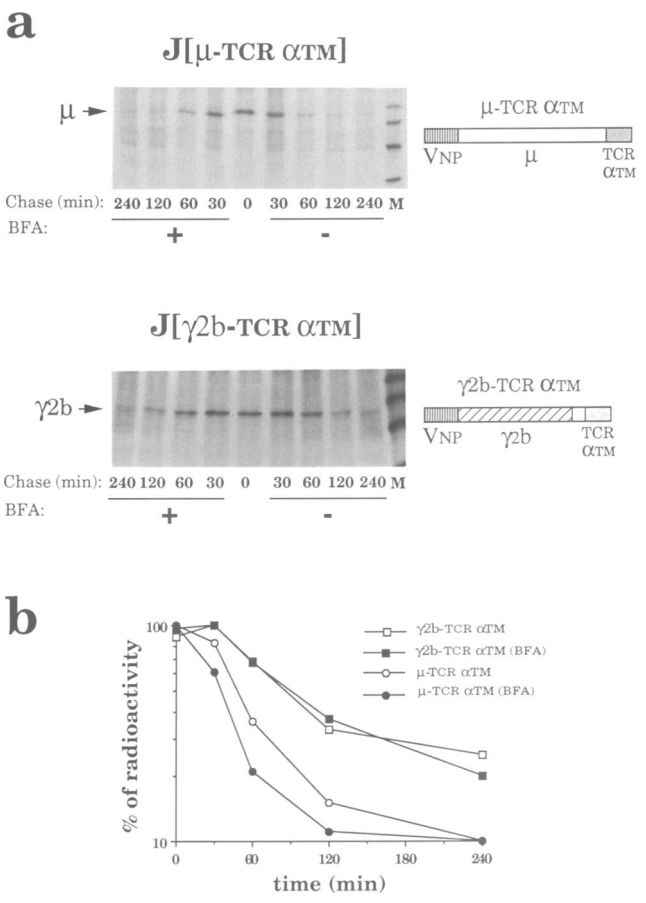

Fig. 6. IgG2bs are more resistant to pre-Golgi proteolysis than IgMs. Pulse-labelled J[μ-TCRαTM] and J[γ2b-TCRαTM] cells were chased for the indicated times in the presence or absence of BFA. (**a**) Cell lysates were immunoprecipitated by NP-cap Sepharose and resolved by SDS–PAGE (10%). Lane M shows the migration pattern of molecular weight markers (phosphorylase B, 97 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa). The structure of the plasmids used to transfect J558L cells is schematized on the right. See Materials and methods for details. (**b**) The graph shows the percentage of H chain associated radioactivity remaining at the different chase points, as determined by densitometric scanning of the gels shown in panel a. Neither μ nor γ2b chains were detected in the supernatants.

mechanism of intracellular retention. First, as in the case of IgM and IgG-μtp chimeric molecules, replacement of Cys575 with serine restores transport through the Golgi. Second, secretion of monomeric CDMμtpCys can be induced by treatment with 2-ME. Third, the protease activities of CDMμtpCys and CDMμtpSer are comparable, ruling out gross folding alterations as the cause of transport incompetency. Taken together, these results indicate that disulfide interchange reactions involving Cys575 are responsible for the intracellular retention of assembly intermediates. Retention is competed by oligomerization or by treatment with 2-ME. Accordingly, CDMμtpCys dimers are the predominant form constitutively secreted by COS-7 transfectants. A similar interrelation between assembly and secretion has been shown for human acetylcholinesterase (Kerem *et al.*, 1993).

The rapid, BFA-insensitive degradation of CDMμtpCys underscores the independence of the pre-Golgi and lysosomal proteolytic pathways. In the endolysosomal compartment, cathepsin D undergoes pH dependent proteolytic cleavages which yield the mature form, consisting of two chains of ~33 and ~14 kDa respectively, linked by a disulfide bond. This molecule is stable for more than 18 h within lysosomes (Hasilik and Neufeld, 1980). By contrast, CDMμtpCys is degraded with a half-life of ~2 h without accumulation of detectable intermediates.

Linking retention to degradation: colocalized determinants

In a simplistic model, retention might determine degradation by keeping the target protein in the same compartment where protease(s) reside. However, as different proteins have different degradation rates (Bonifacino and Lippincott-Schwartz, 1991), ER retention *per se* does not seem to be sufficient to signal degradation. In keeping with this, constructs of cathepsin D which are retained in the ER by the KDEL signal are not degraded (Figure 3). Similarly, constructs lacking the C-terminal cysteine (IgMala575 and CDMμtpSer) are stable also when their transport to the Golgi is blocked by BFA, a condition which does not inhibit degradation. Thus, despite the possibility exists that BFA- or KDEL-mediated retention/retrieval do not lead to colocalization with newly synthesized, transport incompetent proteins which are substrates of pre-Golgi degradation, the existence of 'degradation targeting signals' is at present the best model to explain the exquisite specificity of ER degradation (Bonifacino *et al.*, 1990a,b). The data reported here suggest that Cys575 can indeed act as such a signal. Monomeric IgMs lacking Cys575 are not degraded, indicating that oligomerization is not necessary for stability. Thus, not all assembly events lead to stabilization, but only those which mask specific degradation targeting signals. As it has been described for cytosolic proteins (Pontremoli and Melloni, 1986; Olson and Dice, 1989), such signals could either act as sorting devices to a degradative subcompartment, or directly mediate the binding to non-compartmentalized proteases.

When extended with two independent sequences, the TCR-αTM region and the μtp containing the critical cysteine, both γ2b and μ heavy chains are retained efficiently in the ER, but the latter are more rapidly degraded. Thus, γ2b heavy chains appear to be intrinsically more resistant to ER proteases than μ chains. Hence, the presence of degradation targeting signal(s) is not the only element which determines the rate of protein breakdown.

Where does degradation occur?

At present there is not sufficient evidence to establish the precise intracellular compartment where non-lysosomal degradation of ER synthesized proteins takes place (Bachhawat and Pillai, 1991; Amitay *et al.*, 1992; Stafford and Bonifacino, 1992; Tsao *et al.*, 1992; Wilkström and Lodish, 1992; Shin *et al.*, 1993). As assessed by either immunofluorescence or analysis of their oligosaccharide moieties, neither IgM subunits nor CDMμtpCys reach the Golgi. Furthermore, BFA does not inhibit significantly their degradation. Hence, μtp-driven proteolysis fulfils the requirements of pre-Golgi degradation. Nonetheless, the use of BFA is not sufficient to define a degradative compartment. Treatment with BFA induces resorption of Golgi cisternae into the ER with concomitant redistribution of some Golgi

components. As an example, ectopic processing of oligosaccharide moieties is observed due to the relocation in the ER of some glycosyltransferases (Sampath *et al.*, 1992, see Figure 4b, lane 13). On the other hand, the interpretation of a block of ER degradation by BFA treatment must equally be taken with caution. Based on the fact that BFA inhibits in part IgM degradation, Amitay *et al.* (1992) proposed that in B lymphocytes a post-ER, pre-*trans*-Golgi compartment is the site where non-lysosomal proteases dispose of unassembled secretory IgM. Also in our hands, BFA inhibits in part degradation of IgM subunits in B lymphomas (A.M.Fra *et al.*, unpublished observations), but not in myeloma transfectants. Either a specific pathway exists to handle IgM in B cells, or the latter are exquisitely sensitive to BFA. In B cells, the BFA-induced Golgi resorption might alter significantly the ionic and redox equilibria in the ER, thereby inhibiting degradation of certain substrates.

There are some biochemical requirements that any degradative pathway should meet in order to explain the rapid proteolysis of substrates without accumulation of intermediates. Many retained proteins are endowed with biological activity, and have thus undergone extensive folding and assembly. For instance, transport incompetent CDMμtpCys and IgM monomers display protease and hapten binding activities, respectively. For these substrates, degradation is likely to be facilitated by factors which catalyse unassembly and unfolding. Such a 'reverse' role has recently been proposed for ER chaperones to explain the translocation of certain exogenous toxins to the cytosol (Pelham *et al.*, 1992), an event requiring unfolding (Eilers and Schatz, 1986). That degradation of unassembled TCR subunits does not require exit from the ER has been shown in a semipermeabilized cell system (Stafford and Bonifacino, 1992; Wilkström and Lodish, 1992). How then do nascent proteins, which are bound to express many degradation targeting signals if the latter correspond to assembly and retention surfaces, escape degradation? It is increasingly clear that the ER is a dynamic organelle, subdivided into functionally specialized subcompartments (Sitia and Meldolesi, 1992). Such subregions need not be separated by a membrane, and movement from one to another might not require ATP. Further experiments are required to determine whether a distinct subregion exists within the ER, specialized in proteolytic activity.

Materials and methods

Plasmids

Plasmids pSV-Vμ1 and pSV-VμIn[1,3,4,5]ala (encoding wild type μ and μala575, respectively) have been described previously (Neuberger, 1983; Sitia *et al.*, 1990). Plasmids CDM and CDMK (Pelham, 1988) were kindly given by Dr Hugh Pelham (MRC-LMB, Cambridge, UK). Both constructs encode a human cathepsin D cDNA tagged at the C-terminus by the insertion of the c-myc-derived epitope recognized by the monoclonal antibody 9E10 (Evan *et al.*, 1985). In CDMK, an *Eco*RI fragment encoding the C-terminal SEKDEL hexapeptide is present. To obtain CDMμtpCys, the *Eco*RI fragment of CDMK was replaced by a synthetic double-stranded fragment encoding the μtp, obtained by annealing the following oligonucleotides:

AATTTGAAACCCACACTGTACAATGTCTCCCTGATCATGTCTGACACAGGCGGCAC-CTGCTATTAGATCTAG

and

AATTCTAGATCTAATAGCAGGTGCCGCCTGTGTCAGACATGATCAGGGAGACAT-TGTACAGTGTGGGTTTCA.

The insertion does not recreate the 5′ *Eco*RI restriction site. CDMμtpSer was obtained by site-directed mutagenesis (using an oligonucleotide-directed *in vitro* mutagenesis system, Amersham) using the *Hin*dIII–*Eco*RI fragment of CDMμtpCys cloned in M13mp19 as a template and the oligonucleotide ATCTAATACGAGGTGCCG.

For the construction of pSV-VμTCRαTM the sequence encoding the last 14 amino acids of the luminal domain and the whole transmembrane region and cytoplasmic tail of the TCR α subunit (TCRαTM) was first amplified by PCR from a plasmid encoding the α chain of the 2B4 murine T cell hybridoma (Bonifacino *et al.*, 1990a,b), kindly provided by J.S.Bonifacino (NIH, Bethesda, MD). The oligonucleotides used, TGTGATGCCATGA-TCACCGAGAAA and TGATAGATCTTGGGAGTCAGGCT, inserted at the edges of the amplification product restriction sites for *Bcl*I and *Bgl*II, respectively. These sites were subsequently used for cloning the fragment into the *Bcl*I site located in the μtp sequence of pSV-V[In 1,3,4,5] (Neuberger and Williams, 1988). The C-terminal amino acid sequence of the protein encoded by pSV-VμTCRαTM is GKPTLYNVSLITEKSFETDMNLNF-QNLSVMGLRILLLKVAGFNLLMTLRLWSS.

To construct pSV-Vγ2bTCRαTM the *Bcl*I–*Pvu*I fragment containing the TCRαTM was purified from pSV-VμTCRαTM and ligated to a *Pvu*I–*Bcl*I fragment derived from pSV-Vγ2b/μtp (Sitia *et al.*, 1990), encoding the heavy chain γ2b extended by the μtp sequence. The C-terminal amino acid sequence of γ2bTCRαTM is identical to that of μTCRαTM shown above.

Cell lines and transfections

Plasmacytomas J558L (Oi *et al.*, 1983) and NS0 (Galfré and Milstein, 1981) and the monkey fibroblast cell line COS-7 (Gluzman, 1981) were maintained in DMEM supplemented with 10% fetal bovine serum. J558L and NS0 cells were transfected with pSV-VμTCRαTM and pSV-Vγ2bTCRαTM by electroporation and stable transfectants selected in medium containing 10 μg/ml mycophenolic acid, 13.6 μg/ml hypoxanthine and 250 μg/ml xanthine. The characteristics of the N[μ1], J[μ1] and J[μala575] transfectants were described previously (Sitia *et al.*, 1987, 1990). Transfectants were expanded in medium supplemented with mycophenolic acid, hypoxanthine and xanthine.

COS-7 cells were transfected with CDM and derivatives by the calcium phosphate precipitation method (Chen and Okayama, 1987) and analysed between 40 and 60 h after transfection by immunofluorescence, biosynthetic labelling or Western blotting.

Biosynthetic labelling, immunoprecipitation and gel electrophoresis

In pulse–chase experiments cells were pre-incubated for 30 min in either methionine or methionine/cysteine-free medium supplemented with 10% dialysed fetal bovine serum and then labelled for 15 min with 0.5 mCi/ml [^{35}S]methionine (New England Nuclear, specific activity >800 Ci/mmol) or Tran^{35}S-label (ICN Radiochemicals), respectively. Labelled cells were then washed and incubated in regular culture medium for various time periods. Unless otherwise indicated, brefeldin A (Sigma) was added at the beginning of chase at 1 μg/ml. At each time point, cells were lysed in 150 mM NaCl, 20 mM Tris pH 7.5, 0.25% NP40, 20 mM iodoacetamide, 1 mM PMSF. Cell lysates and supernatants containing μ chains were precleared with protein A–Sepharose (Pharmacia, Uppsala, Sweden) and immunoprecipitated with affinity-purified rabbit anti-mouse μ and protein A–Sepharose. Following a preclearing step of cell lysates with Sepharose beads conjugated to fetal bovine serum proteins, IgMTCRαTM and IgG2bTCRαTM were affinity-purified using Sepharose-conjugated 4-hydroxy-3-nitrophencetyl (NP-cap, Argon and Milstein, 1984). Precipitation of cathepsin D and its derivatives was performed either with the 9E10 anti-c-myc monoclonal antibody and protein G–Sepharose or with a polyclonal rabbit anti-human cathepsin D (kindly given by Dr Ciro Isidoro, University of Turin, Italy), followed by protein A–Sepharose. After extensive washing, immunoprecipitates were analysed by SDS–PAGE. Gels were fixed and impregnated with Amplify (Amersham) before exposure to Kodak X-Omat films. For quantification, fluorographs were scanned by a computing densitometer (Molecular Dynamics).

Western blotting

Samples containing cathepsin D chimeric proteins immunopurified by Sepharose-bound anti-myc antibodies were resolved on SDS–polyacrylamide gels (10%) under reducing conditions before electroblotting on to nitrocellulose filters in a buffer consisting of 25 mM Tris, 190 mM glycine and 20% methanol. Blots were first saturated with 3% non-fat dried milk in phosphate buffered saline containing 0.05% Tween-20 (Sigma) and then incubated for 1 h with a polyclonal rabbit anti-cathepsin D followed by a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed) in the presence of 1% normal mouse serum. Both antibodies were diluted in phosphate buffered saline containing 0.05% Tween-20. Bands were detected by enhanced chemiluminescence (ECL Western blotting, Amersham) according to the manufacturer's instructions.

Acknowledgements

The expert technical assistance of E.Pasqualetto is gratefully acknowledged. We thank Drs J.S.Bonifacino, A.Ceriotti, C.Isidoro, R.D.Klausner, J.Meldolesi, C.Milstein, M.S.Neuberger, H.R.B.Pelham and C.Valetti for providing reagents and for helpful suggestions, criticism and discussions, L.Cremonesi and N.Soriani for providing synthetic oligonucleotides and C.DeMarchi for secretarial assistance. This work was in part supported through grants from Ministero della Sanità (AIDS Special Project), Associazione Italiana per la Ricerca sul Cancro (AIRC) and CNR Target Projects on Biotechnology and Bioinstrumentation and Genetic Engineering. A.M.F. is the recipient of a fellowship from AIRC.

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Received on June 7, 1993; revised on August 8, 1993