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The Differential Effects of Dithiothreitol and 2-Mercaptoethanol on the Secretion of Partially and Completely Assembled Immunoglobulins Suggest that Thiol-mediated Retention Does Not Take Place in or Beyond the Golgi

Caterina Valetti*†‡ and Roberto Sitia*§‖

*DIBIT-San Raffaele Scientific Institute, 20132 Milano, Italy; †Istituto di Anatomia, Università di Genova, 16132 Genova, Italy; and §Istituto Nazionale per la Ricerca sul Cancro, 16132 Genova, Italy

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Dithiothreitol (DTT) blocks the endoplasmic reticulum (ER)-Golgi transport of newly synthesized immunoglobulin (Ig) molecules, whereas 2-mercaptoethanol (2ME) allows secretion of unpolymerized Igs otherwise retained intracellularly by disulphide interchange reactions. To understand this dichotomy, we have compared the effects of DTT and 2ME on the assembly, intracellular transport, and secretion of a panel of chimeric Igs that are either constitutively secreted or retained intracellularly. Our results demonstrate that DTT, but not 2ME, reduces some of the inter- and intrachain disulphide bonds and causes partial disassembly of H2L2 complexes and unfolding of individual chains in the ER. Upon DTT removal, heavy (H) and light (L) chains reform hapten-binding H2L2 molecules, which are later secreted. Reduction of the H2L2 interchain disulphide bonds can occur along the entire secretory pathway; however, in or beyond the Golgi this does not result in efficient H-L disassembly or unfolding. As a consequence, DTT does not block the exit from the Golgi. Moreover, unpolymerized Igs—normally retained in a pre-Golgi compartment—no longer require reducing agents to be secreted once they have reached the Golgi. Thus, little if any thiol-mediated retention seems to take place in or beyond the Golgi complex.

INTRODUCTION

In eukaryotic cells the endoplasmic reticulum (ER)[1] is the port of entry of newly synthesized proteins destined to the exocytic compartment. It is in the ER that membrane and secretory proteins begin to fold, assemble, and oligomerize. Although the three-dimensional structure of proteins is dictated by their aminoacid sequences (Anfinsen, 1973), the processes of folding, assembly, and oligomerization are catalyzed in vivo (Gething and Sambrook, 1992). The ER is also endowed with quality control functions, by which molecules that fail to attain the proper tertiary and quaternary structure are retained and eventually degraded (Hurtley and Helenius, 1989; Pelham, 1989; Klausner and Sitia, 1990; Fra *et al.*, 1993). The rich array of chaperone molecules residing in the ER is thought to play a major role in these processes (Helenius *et al.*, 1992).

Immunoglobulins (Igs) offer a powerful experimental model to investigate the molecular mechanisms that link quality control, structural maturation, and intracellular transport of newly made proteins. As for many proteins that are translocated into the ER, the folding of nascent Ig heavy (H) chains starts

‡ Present address: Department of Cell Biology, University, Science III, CH-1211 Genève, Switzerland.
‖ Corresponding author.

Abbreviations used: 2ME, 2-mercaptoethanol; DTT, dithiothreitol; ER, endoplasmic reticulum; Ig, immunoglobulin; H, heavy chains; L, light chains; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

cotranslationally from the N-terminal domain and proceeds vectorially toward the C-terminal domains. This vectoriality might be important to prevent aberrant folding and disulphide bond formation (Bergman and Kuehl, 1979a,b). Once folding of at least the variable (VH) and first constant (CH1) domains is completed, assembly with preexisting light (L) chains proceeds rapidly along predetermined pathways, which vary depending on the isotype. In the mouse IgM and IgG2b follow the H-HL-(H2L)-H2L2 pathway, whereas IgG1 are assembled in the H-H2-H2L-H2L2 order (Scharff *et al.*, 1970). In the absence of L chains, H chains interact with BiP, one of the major ER chaperones, and remain therefore stuck in the ER lumen unless the CH1 is deleted (Hendershot *et al.*, 1987). Assembly with L chains displaces BiP and allows Ig secretion (Hendershot, 1990). Because only H2L2 molecules are secreted, mechanisms preventing the transport of HL molecules are also implied.

Only pentameric or hexameric IgM are secreted by plasmacells (Davis *et al.*, 1989b; Sitia *et al.*, 1990; Randall *et al.*, 1992). Thus, unlike IgG or other monomeric Igs, μ2L2 must further assemble to be transported through the Golgi. The C-terminal cysteine of the secretory μ chain (Cys575) is responsible for both assembly and retention of unpolymerized μ2L2 subunits. When the last 20 residues of the secretory μ chain (the μ tailpiece [μtp]) containing Cys575 are added to the C terminus of γ2b chains, chimeric IgG2bμtpCys are retained intracellularly. Replacing the critical cysteine residue with serine restores secretion (Sitia *et al.*, 1990). Thus, at least two elements in the μ chain—the CH1 and Cys575—are involved in the sequential events that underlie the quality control of secretory IgM, to verify H-L assembly and μ2L2 polymerization, respectively. The role of disulphide interchange reactions in intracellular retention is further supported by the observation that certain reducing agents (i.e., 2-mercaptoethanol [2ME] and N-acetyl cysteine) induce secretion of unpolymerized IgM and of IgG2bμtpCys (Alberini *et al.*, 1990). However, another reducing agent, dithiothreitol (DTT), has been shown to cause the selective retention of some newly synthesized proteins in the ER (Braakman *et al.*, 1992a,b; Kaji and Lodish, 1993a,b; Lodish and Kong, 1993).

To understand this dichotomy, we have now compared the effects of DTT and 2ME on a panel of chimeric Ig molecules that are either retained by disulphide interchange reactions or not. By labeling proteins in the ER or in the Golgi, it has been possible to investigate independently assembly, oligomerization, and quality control along the secretory pathway. Our results demonstrate that Ig folding and assembly need not be cotranslational and that the thiol-mediated retention mechanisms, so efficient in the ER, become of little if any importance once the proteins have reached the Golgi.

MATERIALS AND METHODS

Cell Lines

J[μ1], J[γ2b-μtpCys], and J[γ2b-μtpSer] were originated by transfecting J558L (a λ chain–producing myeloma) with plasmids pSV-Vμ1, pSV-Vγ2b-μtpCys, and pSV-Vγ2b-μtpSer (Neuberger, 1983; Sitia *et al.*, 1990). Briefly, pSV-Vμ1 encodes a wild-type μ chain gene; in pSV-Vγ2b-μtpCys the murine γ2b constant gene was extended with the 20 carboxy terminal residues of secretory μ chains, the μtp. In pSV-Vγ2b-γtpSer the C-terminal cysteine was replaced by Ser. Cells were maintained in Dulbecco's minimal essential medium (DME) supplemented with 1 mM glutamine, penicillin, and streptomycin, 5% fetal calf serum (FCS), 5 μg/ml mycophenolic acid, 13.6 μg/ml hypoxanthine, and 0.25 mg/ml xanthine.

Endogenous Labeling

For pulse-chase experiments cells were preincubated for 15 min in either methionine or methionine/cysteine-free medium supplemented with 2% dialyzed FCS and 1 mM glutamine and then labeled at 2×10^7/ml for 5-30 min with 1 mCi/ml [^{35}S]methionine (New England Nuclear, Firenze, Italy) (specific activity >800 Ci/mmol) or Tran[^{35}S]label (ICN Radiochemicals, Milano, Italy) (specific activity >1000 Ci/mmol), respectively. Pulse-labeled cells were washed and incubated either in regular culture medium or in presence of 2-ME or DTT for various time periods. In one experiment cells were pulsed for 5 min in the presence or absence of 5 mM DTT and chased as above.

For galactose-labeling experiments cells were preincubated for 30 min in glucose-free RPMI containing 2% dialyzed fetal bovine serum and 1 mM glutamine, pulse-labeled for 15 min with 100 μCi/ml [^{14}C(U)] D-galactose (New England Nuclear) (specific activity 308.5 mCi/mmol), washed in DME medium containing unlabeled glucose and galactose in excess (3.5- and 20-fold, respectively), and chased in the same medium with or without DTT for the indicated times.

As determined by trypan blue exclusion and analyses of lactate dehydrogenase activity (Rubartelli *et al.*, 1990) in the supernatants of cells chased in the presence of reducing agents, cell viability was always ≥95% for DTT concentrations up to 4 mM. At each time point of chase, samples were chilled and centrifuged for 5 min at $1000 \times g$. Either iodoacetamide or N-ethylmaleimide (both at the final concentration of 20 mM) were added to the supernatants to block the formation of disulphide bonds. Cells were washed once in ice-cold phosphate-buffered saline (PBS) before lysis in 0.5% NP-40, 150 mM NaCl, 50 mM tris(hydroxymethyl) aminomethane (Tris)-HCl pH 7.5, 5 mM EDTA, and either 20 mM iodoacetamide or 20 mM *N*-ethylmaleimide. 2-ME or DTT were present in the washing buffer at the concentration used in the chase. To minimize proteolysis, phenylmethylsulphonyl fluoride (PMSF) (1 mM) was added to both cell lysates and supernatants. When the chase periods were short, an identical volume of a twofold concentrated lysis buffer, containing N-ethylmaleimide, was directly added to the samples without separating the cells from the medium.

Immunoprecipitation and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

When necessary, samples were stored at −20°C before immunoprecipitation. Cell lysates and supernatants were first precleared by rotating for ≥45 min at 4°C with sepharose beads conjugated to FCS and immunoprecipitated with affinity-purified class specific antibodies (goat anti-mouse γ and goat anti-mouse μ) or goat anti-mouse λ antibodies (Southern Biotechnology, Birmingham, AL) followed by protein-A sepharose (Pharmacia, Uppsala, Sweden). Sepharose-bound (4-hydroxi-3-nitrophenyl) acetyl (NP) was used to selectively precipitate HL assemblies (Reth *et al.*, 1979; Valetti *et al.*, 1991). In some experiments the leftovers from the first cycle of immunoprecipitation were sequentially precipitated with a second antibody.

After washing three times in 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% NP-40 and once in 5 mM Tris-HCl pH 7.5, the immunoprecipitates were eluted at 95°C for 2 min in 5% SDS and analyzed by reducing and nonreducing SDS-PAGE. [^{14}C]-labeled molecular weight markers (myosin, 200 kDa; phosphorylase B, 97 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; and carbonic anhydrase, 30 kDa) were purchased from Amersham Italia (Milan, Italy). To prepare reduced and oxidized λ chain markers, methionine-labeled IgM and IgG2b were immunopurified from the supernatants of J[μ1] and J[γ2b-μtpSer], respectively. Mild reduction (2 mM DTT for 10 min at 37°C) generates λ chains that maintain both of the intrachain disulphide bonds (λox), whereas reduced λ chains (λred) are obtained by incubation for 5 min at 95°C with 50 mM DTT and 1% SDS, followed by alkylation with 150 mM N-ethylmaleimide.

Gels were fixed and impregnated with Amplify (Amersham, Arlington Heights, IL) before exposure to Kodak (Rochester, NY) X-Omat films. For quantitative analyses fluorographs were scanned by a computing densitometer (Molecular Dynamics, Sunnyvale, CA). At least two exposures of each gel were analyzed.

Analysis of IgM Under Nondenaturing Conditions

Aliquots of the NP-40 lysates of galactose-labeled J[μ1] cells were applied onto 5 ml 5–20% linear sucrose gradients in PBS. Purified polymeric and monomeric IgM were used as markers on separate gradients (Sitia *et al.*, 1990). After centrifugation for 4 h at 40 000 rpm on a SW 50.1 rotor (Beckman Instruments, Fullerton, CA), fractions were collected from the bottom of the tube. Aliquots from the individual fractions were analyzed for the presence of trichloroacetic acid–precipitable radioactivity or immunoprecipitated with NP-sepharose (Valetti *et al.*, 1991) and resolved by SDS-PAGE (3–14% gradients) under nonreducing conditions. The number of fractions collected varied in the different samples (23 in the case of cells chased for 10 min with DTT and 10 min without DTT, and 27 for cells chased for 20 min with DTT). However, when the cumulative volumes are compared, the peaks corresponding to polymers and monomers migrated similarly in the two gradients. The three fractions corresponding to polymers were pooled, whereas those corresponding to smaller material were analyzed individually.

Endoglycosidase H Digestion

Endoglycosidase H was purchased from Boehringer Mannheim (Mannheim, Germany). Radiolabeled samples were precipitated with NP-sepharose or anti-μ and protein A-sepharose and washed as described above. During the last wash beads were divided into two aliquots, resuspended in 20 μl of 0.08% SDS and 100 mM 2ME, heated at 70°C for 10 min, and chilled. Ten microliters of a buffer containing 250 mM Na-citrate pH 5.5, 2 mM PMSF were added, with or without 1 mU of the enzyme. After overnight incubation at 37°C, samples were eluted in Laemmli buffer and resolved on SDS-PAGE under reducing conditions.

Immunofluorescence

Immunofluorescence was performed as described previously (Sitia *et al.*, 1987). Briefly, cells were allowed to bind onto poly-L-lysine–coated slides (≥30 min at 4°C), fixed with 3.7% formaldehyde in PBS (10 min at 4°C), permeabilized with 0.05% Triton X100 in PBS (10 min at room temperature), and stained with optimal dilutions of fluorescent antibodies or lectins in PBS containing 10 mM NaN3 and 0.02% gelatin. Fluorescein- or rhodamin-conjugated goat anti mouse-γ2b were obtained from Southern Biotechnology. Rat anti mouse BiP (Bole *et al.*, 1986), a generous gift of Dr. L. Hendershot (Memphis, TN), was used in indirect immunofluorescence followed by fluoresceinated goat anti-rat antibodies (Cappell, Cochranville, PA) that had been preadsorbed with sepharose beads conjugated to mouse immunoglobulins. Fluoresceinated ricin-A, a lectin that binds to galactose and can be used as a marker of the transGolgi (Nilsson *et al.*, 1993), was purchased from Sigma (St. Louis, MO) and used according to the manufacturer's instructions. Samples were inspected in a Zeiss (Thornwood, NY) LSM confocal scanning microscope and photographed with Kodak T-MAX 100 films.

RESULTS

Different Effects of DTT and 2ME on Secretion of Chimeric IgG Molecules

To compare the effects of 2ME and DTT on the intracellular transport of secretory molecules, we made use of chimeric IgGs in which the C-terminal end of γ2b chains had been extended by the μ tailpiece (μtp) with either a cysteine (IgG2b-μtpCys) or a serine (IgG2b-μtpSer) in the penultimate position. The former are retained intracellularly, whereas the latter are constitutively secreted (Sitia *et al.*, 1990). As previously reported (Alberini *et al.*, 1990), 5 mM 2ME induces secretion of

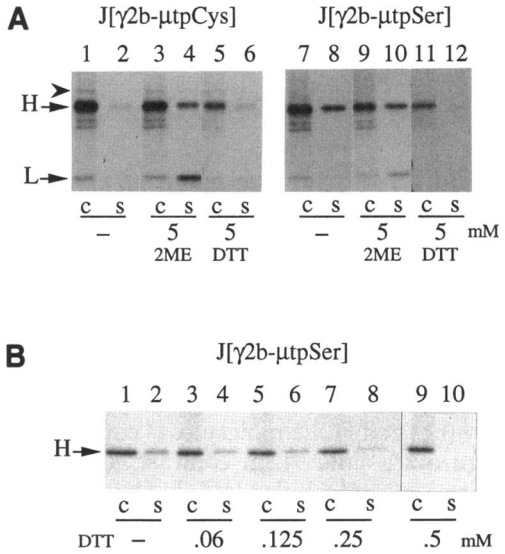
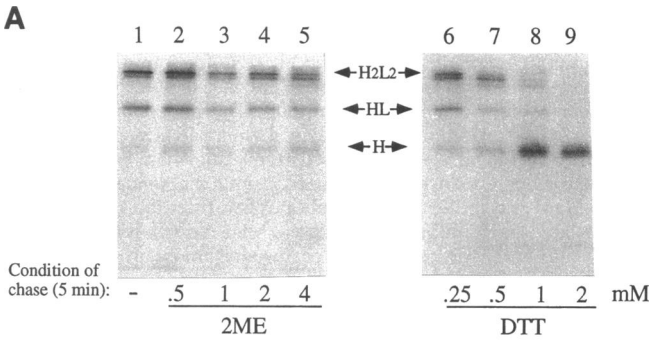


Figure 1. Different effects of DTT and 2ME on IgG secretion by myeloma transfectants. (A) J[γ2b-μtpCys] and J[γ2b-μtpSer] cells were pulsed for 15 min with [^{35}S]-methionine and chased for 2 h with or without 5 mM 2ME or DTT. Cells were washed once in ice-cold PBS containing 10 mM sodium azide before lysis in NP40 and 20 mM iodoacetamide. Cell lysates (c) and supernatants (s) were immunoprecipitated by goat anti mouse-γ and protein A-sepharose. Immunoprecipitates were resolved on 10% polyacrylamide gels under reducing conditions. The increased intensity of the 25-kDa band in lanes 4 and 10 is due to the fact that, unexpectedly, several reducing agents, including 2ME, DTT, and GSH, induce some cross-reactivity of λ chains with protein A-sepharose. Arrows on the left hand margin indicate the migration of H and L chains. A 78-kDa band probably consisting of BiP/grp78 coprecipitates with anti-γ in J[γ2b-μtpCys] (see arrowhead) but is not evident in the J[γ2b-μtpSer] cell lysates. (B) Titration of the inhibitory effects of DTT on IgG secretion. J[γ2b-μtpSer] cells were pulsed and chased as above in the presence of the indicated concentrations of DTT. Anti-γ immunoprecipitates from the cell lysates (c) and from the supernatants (s) were resolved on a 10% polyacrylamide gel under reducing conditions. Only the heavy chain region of the gels is shown.

IgG2b-μtpCys (Figure 1A, compare lanes 2 and 4), whereas it has little effects on IgG2b-μtpSer (Figure 1A, lanes 8 and 10). In contrast, the same concentration of DTT not only fails to induce IgG secretion by J[γ2b-μtpCys] cells (Figure 1A, lane 6) but actually inhibits constitutive secretion of IgG2b-μtpSer (Figure 1A, lane 12). When both 2ME and DTT are added simultaneously, the negative effects of DTT are dominant, and Ig secretion is inhibited. A titration of the inhibitory effects of DTT is shown in Figure 1B. Already at the concentration of 0.06 mM there is some inhibition; at 0.25 mM DTT secretion of IgG2b-μtpSer is reduced more than twofold and at 0.5 mM the block is virtually complete. Thus, DTT inhibits secretion independently from the retention by an active μtp sequence.

In both J[γ2b-μtpCys] and J[γ2b-μtpSer] transfectants, DTT and, to a lesser extent, 2ME induce some intracellular degradation of newly synthesized Igs. However, densitometric analyses reveal that the secretory block induced by DTT is not only due to degradation. For instance at 0.5 mM DTT, secretion is inhibited more than 95%, whereas intracellular degradation does not exceed 40% (Figure 1B, compare lanes 9 and 10 with 1 and 2).



B

	-	2ME	DTT			
[mM]	-	4	0.25	0.5	1	2
% γchains precipitated by NP-Sepharose	100	62	67	55	10	8

Figure 2. Unlike 2ME, DTT reduces newly formed interchain SS bonds. (A) J[γ2b-μtpSer] cells were pulsed for 15 min with [^{35}S]-methionine and chased for 5 min with the indicated concentrations of 2ME (lanes 2–5) or of DTT (lanes 6–9) before lysis in the presence of NP40 and iodoacetamide and immunoprecipitation (anti-γ). Fluorogram of a SDS-PAGE (8%) run under nonreducing conditions. Arrows indicate the migration of H2L2, HL, and H chains. (B) Aliquots of the samples shown in A were also precipitated by NP-sepharose, and fluorograms of the gels scanned by a computing densitometer. The data indicate the percentage of γ associated radioactivity precipitated by NP-sepharose, relative to the amount precipitated by anti-γ.

DTT, but not 2ME, Reduces the Interchain Disulphide Bonds

How can the two reducing agents have different effects? Unlike 2ME, DTT, a stronger, divalent reducing agent, might reduce some of the disulphide bonds already formed between (and within) H and L chains, thereby causing partial disassembly and unfolding. That this is indeed the case is shown in Figure 2A. Reduction of newly synthesized H2L2 molecules is complete at 2 mM DTT (Figure 2A, lane 9). In contrast, 4 mM 2ME has little effects on intracellular IgG (Figure 2A, lane 5).

To monitor the extent of H-L dissociation induced by DTT, aliquots of the samples were precipitated with NP-sepharose, a reagent that selectively recognizes VHVL assemblies, regardless of the presence of an interchain disulphide bond between the constant domains (Valetti *et al.*, 1991). The results shown in Figure 2B indicate that DTT induces H-L disassembly to a greater extent than 2ME. However, it should be noted that after a 5-min treatment with 0.5 mM DTT (a concentration sufficient to block Ig secretion almost completely) (Figure 1B), not all H2L2 molecules are dissociated.

H2L2 Reassembly After Removal of DTT

That DTT induces H2L2 disassembly is confirmed by pulse chase experiments (Figure 3). When pulse-labeled J[γ2bμtpSer] cells are chased in the absence of DTT, anti-λ antibodies precipitate three main bands, consisting of L, L2, and H2L2, respectively (Figure 3B, lanes 1 and 3). Traces of an HL intermediate are also detectable in the lysates of cells chased for 30 min (Figure 3B, lane 1). As expected, λ chain homodimers HL and H2L2 are not present in the lysates of cells chased for the same period in the presence of DTT (Figure 3B, lane 2). Moreover, only traces of γ chains (50–55 kDa) are precipitated by anti-λ, suggesting that most H2L2 complexes are disassembled into free γ chains. Indeed, the latter can be precipitated by anti-γ antibodies from the anti-λ leftovers (Figure 3C, lanes 2 and 4). These free γ chains readily reassemble into H2L2 complexes (Figure 3B, lane 5) that can be secreted (Figure 3D, bar 5) when DTT is washed out and cells are chased for further 90 min. Also the secretion of free λ chains is inhibited by DTT, although less than that of IgG (Figure 3, compare D and E, bar 4). Taking into account that DTT induces some intracellular degradation of newly made IgG (see Figure 1), these results demonstrate that the DTT-induced secretory block is reversible and imply that Ig assembly can occur posttranslationally.

Posttranslational Folding of Ig λ Chains

The λ chain band appears as a doublet in DTT-treated samples (Figure 3B, lanes 2 and 4). This is particularly evident in Figure 3C, where small amounts of L chains are detected, probably because of incomplete precipitation in the first cycle with anti-λ antibodies. This doublet is not detected in gels run under reducing con-

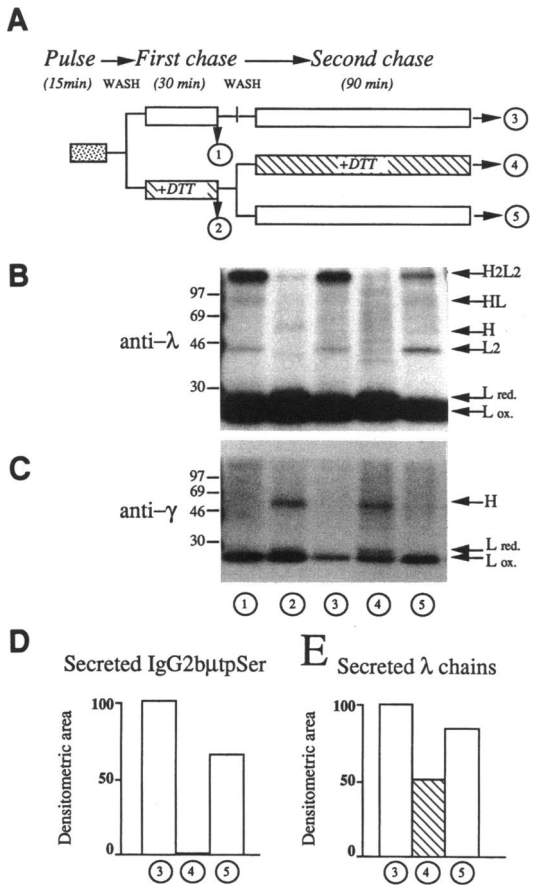


Figure 3. The DTT-induced secretory block is reversible. (A) Outline of the experimental scheme employed. J[γ2b-μtpSer] cells were pulsed for 15 min with [^{35}S]-methionine and cysteine, chased for 30 min in the absence or presence of 2 mM DTT (1 and 2, respectively), and washed and cultured for an additional 90 min without (3 and 5) or with 2 mM DTT (4). Circled numbers are used to identify the lanes of the gels shown in B and C and the bars in the histograms shown in D and E. (B and C) Cell lysates were sequentially immunoprecipitated with anti-λ (B) and anti-γ (C). The amount of residual λ chains precipitated by anti-γ and protein A-sepharose in C did not exceed 5% of what is present in the corresponding lanes of B and probably originates from incomplete precipitation of anti-λ immune complexes by protein A-sepharose. Fluorogram of gels (8%) run under nonreducing conditions. The migration of molecular weight markers (see MATERIALS AND METHODS) and of H2L2, HL, H, L2, and monomeric L chains, both in their oxidized (L ox.) and partially reduced (L red.) form, is indicated on the margins. (D and E) Secreted IgG2bμtpSer (D) and λ chains (E) were quantitated by densitometric analyses of reducing gels. The results are expressed as the percentage of γ- or λ-specific radioactivity present in the supernatants relative to cells chased without reducing agents. For the identification of numbers below bars see A. All λ chains secreted in the presence of DTT migrate as the fully oxidized form, suggesting that the reversible inhibition of their secretion correlates with reduction of the intrachain SS bond(s) and partial unfolding.

ditions, suggesting that DTT reduces at least one of the two intrachain SS bonds (Figure 4). The presence of disulphide bonds is known to reduce the hydrodynamic volume of a protein by restricting the flexibility of the peptide backbone (Creighton, 1989). Indeed, λ chains with both the intrachain disulphide bonds (λox) can be readily resolved on nonreducing SDS-PAGE from molecules reduced and alkylated in vitro (λred) (Figure 4A, lane 1, see arrows on the left). This offers a convenient assay for monitoring λ chain folding, which requires both intrachain SS bonds. In agreement with the rapid, cotranslational formation of the latter (Bergman and Kuehl, 1979b), most λ chains synthesized during a 5-min pulse comigrate with λox. Only a faint smear, probably corresponding to a few incompleted and/or partially reduced chains, is detected above the main λ chain band at the end of the pulse (Figure 4A, lane 2) and tends to disappear during the chase (Figure 4A, lanes 3–5). In contrast, the presence of DTT during the pulse results in newly synthesized λ chains that migrate slowly; four discrete bands can be identified (Figure 4A, lane 7). The most abundant one, which comigrates with λred, rapidly chases into the fully oxidized form (λox) when DTT is removed. Two intermediates are also detected, which are likely to correspond to molecules in which only one of the two intrachain bonds is formed (λints). The slower of the two intermediates predominates at all chase times. When resolved after reduction and alkylation (Figure 4B), the same samples yield a single λred band, confirming that the heterogeneity de-

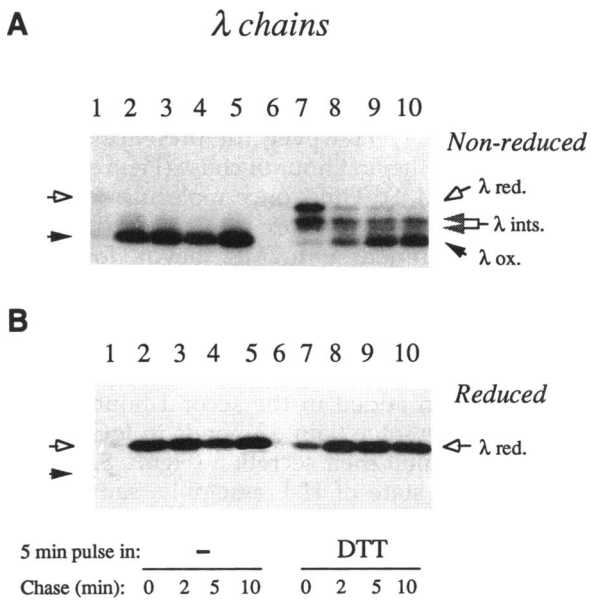


Figure 4. Folding of L chains can occur posttranslationally. λ chain–producing myeloma cells were pulsed for 5 min with [^{35}S]-methionine and cysteine in the presence (lanes 7–10) or absence (lanes 2–5) of 2 mM DTT and chased for the indicated times without DTT before lysis. Aliquots of the anti-λ immunoprecipitates were resolved on SDS-PAGE (12%) under nonreducing conditions (A) or after reduction and alkylation (B). Lane 6 shows the migration of purified reduced and alkylated λ chains (λred, open arrows), whereas equal amounts of fully reduced and oxidized λ (λox, solid arrows) chains are shown in lane 1.

scribed above corresponds to different arrangements of the intrachain disulphides.

These results demonstrate that formation of the two λ intrachain SS bonds is inhibited when DTT is present during translation. Upon removal of DTT, reduced λ chains are rapidly oxidized (Figure 4B, lanes 7–10) and later secreted either as Lox monomers or L2 homodimers, or in cells that express also H chains as H2L2 molecules. Folding and assembly can therefore occur posttranslationally.

Effects of DTT on IgG2b-μtpCys Molecules that Have Been Mobilized by Treatment with 2ME

Unless 2ME is added to the medium, newly synthesized IgG2bμtpCys molecules are retained intracellularly, probably in a pre-Golgi compartment (see below). Previously reported pulse-chase experiments indicate that after 1 h of chase in the presence of 2ME there is still a significant intracellular pool of IgG2bμtpCys molecules on their way to secretion (Alberini *et al.*, 1990). Do molecules which have been mobilized by treatment with 2ME still require the reducing agent to reach the extracellular space? Does DTT block their secretion? To answer these questions, pulse-labeled J[γ2b-μtpCys] cells were chased for 1 h with or without 2ME, washed, and further incubated for an additional hour in the absence or in the presence of 2ME or DTT (Figure 5A). As expected, when cells are chased without 2ME in both chase periods, very little IgG2bμtpCys is detected extracellularly (Figure 5A, lane 2). In contrast, the continuous presence of the reducing agent induces secretion (Figure 5A, lane 4). However, the presence of 2ME is required only in the first hour of chase (Figure 5A, compare lanes 3 and 4). Thus, once molecules have been allowed to begin their journey along the exocytic compartment by treatment with 2ME, they no longer need the reducing agent to complete it. The small increase in secretion induced by 2ME in the second hour of chase is probably due to further mobilization of IgG2bμtpCys molecules from a pre-Golgi compartment (Alberini *et al.*, 1990). When added in the second hour of chase, DTT reduces the interchain SS bonds in IgG2bμtpCys but does not inhibit their secretion (Figure 5A, lane 5). To monitor the state of H-L assembly, samples were precipitated by NP-sepharose (Figure 5B). Although this reagent fails to precipitate some free γ2bμtpCys chains present intracellularly at the end of the pulse (Figure 5B, compare lanes 1A and 1B), it brings down most extracellular γ2bμtpCys chains, either when they are covalently bound to L (Figure 5B, lanes 3–4) or when they migrate in SDS gels as free H (Figure 5B, lane 5). Thus, although the interchain disulphide bonds are reduced by DTT, there is little H-L dissociation of IgG2bμtpCys molecules that have been mobilized by the previous treatment with 2ME. That the washing conditions utilized ensure complete removal of 2ME is

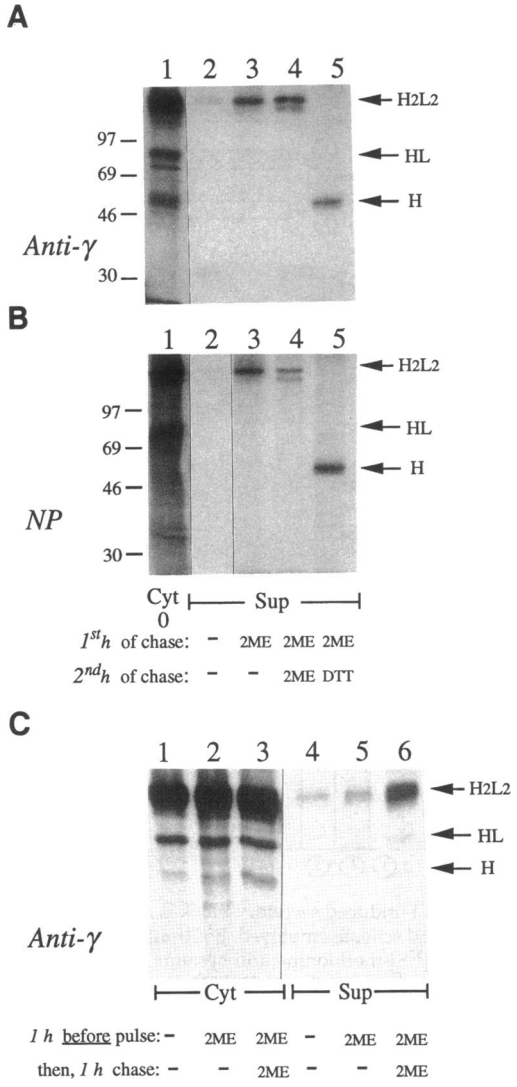


Figure 5. Secretion of IgG2bμtpCys molecules mobilized by 2ME can continue in the absence of reducing agents and is not inhibited by DTT. (A and B) J[γ2b-μtpCys] cells were pulsed for 15 min with [^{35}S]-methionine and cysteine and chased for 1 h with (lanes 3–5) or without (lane 2) 4 mM 2ME. Cells were then washed and cultured for another hour in plain medium (lanes 2 and 3) or in the presence of 4 mM 2ME (lane 4) or of 2 mM DTT (lane 5) as detailed at bottom of B. The supernatants after the second hour of chase (lanes 2–5, Sup) and the lysates of cells harvested immediately after the pulse (lane 1, Cyt) were immunoprecipitated with anti-γ and protein A-sepharose (A) or NP-sepharose (B). Note that only one-half of the sample was loaded onto lane 4 in B. The migration of molecular weight markers is indicated on the left hand margin. As determined by densitometric analyses, maximal IgG2bμtpCys secretion was obtained when 2ME was present also in the second hour of chase (lane 4). Relative to this, 78% was the amount secreted when 2ME was absent in the second hour of chase (lane 3) and 65% in the presence of DTT (lane 5). (C) J[γ2b-μtpCys] cells were cultured for 1 h with (lanes 2, 3, 5, and 6) or without (lanes 1 and 4) 2ME and washed before the pulse with radioactive amino acids. Cells were then chased for 1 h with (lanes 3 and 6) or without 2ME (lanes 1, 2, 4, and 5). Cell lysates (Cyt) and supernatants (Sup) are shown in lanes 1–3 and lanes 4–6, respectively. All panels show fluorograms of SDS-PAGE (8%) run under nonreducing conditions.

shown in Figure 5C. When J[γ2b-μtpCys] cells are cultured for 1 h in 2ME and washed before the radioactive pulse, IgG2b-μtpCys are not secreted (Figure 5C, lane 5) unless the reducing agent is added again (Figure 5C, lane 6).

Taken together, these results indicate that the thiol-based mechanism that retains IgG2bμtpCys does not operate once these molecules have been mobilized by 2ME treatment and that the mobilized oligomers become resistant to DTT-induced disassembly (compare with Figure 2B).

2ME, but not DTT, Induces Movement of IgG2b-μtpCys from the ER to the Golgi

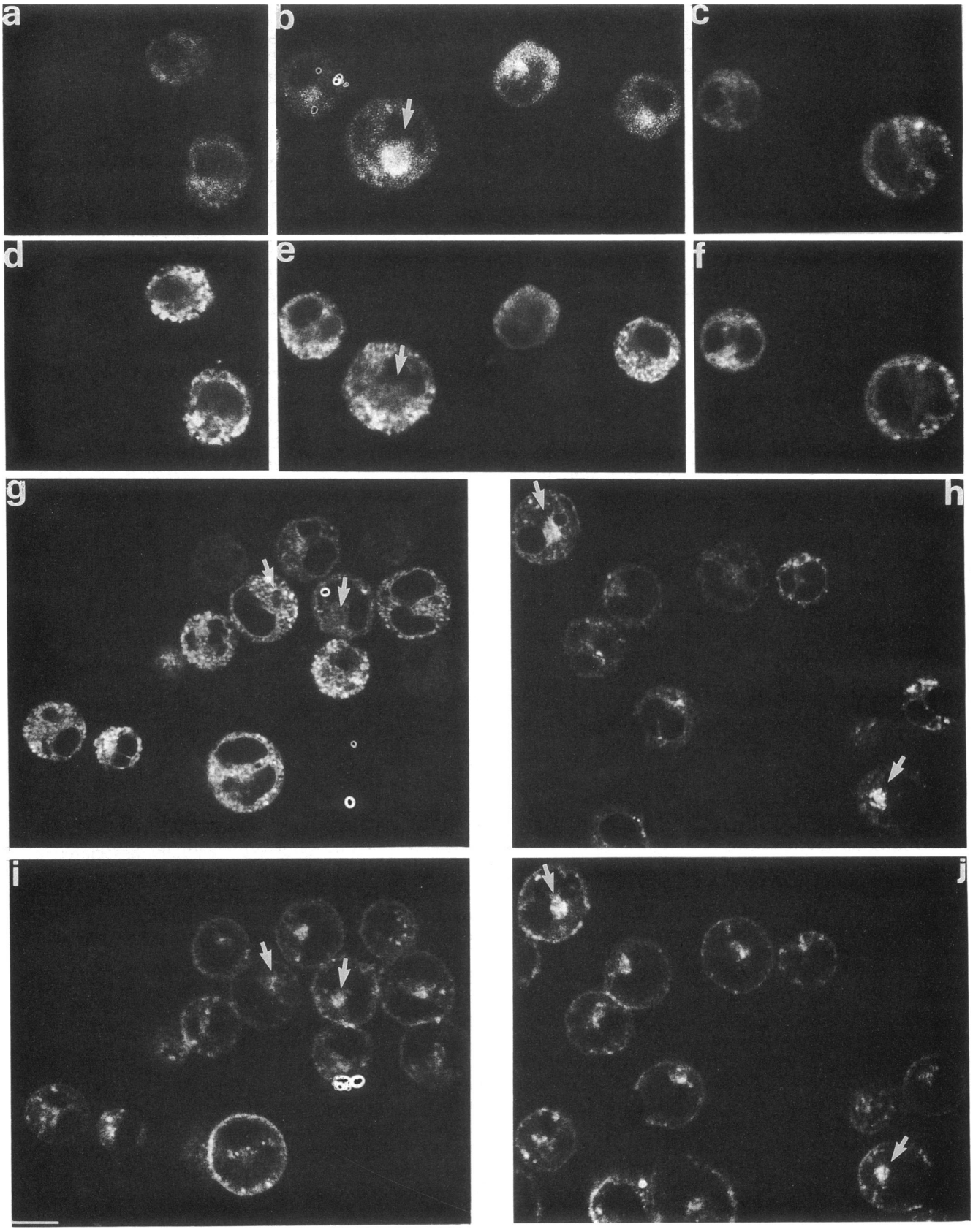
Confocal scanning microscopy was employed to determine the intracellular localization of IgG2bμtpCys molecules before or after treatment with reducing agents (Figure 6). Anti-BiP antibodies and ricin A were used as markers of the ER and the transGolgi, respectively. In untreated J[γ2b-μtpCys] cells, anti-γ antibodies decorate a diffuse reticular region (Figure 6, a and g), largely overlapping with the anti-BiP distribution (Figure 6, compare a and d). After 1 h of culture in the presence of 4 mM 2ME, the anti-γ staining pattern becomes concentrated in a juxtanuclear region that excludes BiP (Figure 6, see arrows on b and e). Costaining with rhodaminated anti-γ and fluorescinated ricin-A (Figure 6, g–j) confirms that in the presence of 2ME some IgG2b-μtpCys can distribute throughout the Golgi. In contrast, exposure to 2 mM DTT does not induce the accumulation of IgG2b-μtpCys outside the ER (Figure 6, c and f). These findings suggest that the 2ME-induced transport to the Golgi may explain both the secretion of mobilized IgG2bμtpCys in the absence of 2ME and their resistance to DTT-induced disassembly (see Figure 5).

DTT Does Not Inhibit the Exit of Galactose-labeled IgM from the transGolgi

This hypothesis was confirmed by galactose-labeling experiments. Owing to the fact that the enzyme galactosyl-transferase is primarily localized in the transGolgi (Nilsson *et al.*, 1993), radioactive galactose can be used to label selectively glycoproteins that have reached this compartment. Because γ2b chains have only one N-linked glycosylation site, IgG2bμtpCys molecules cannot be labeled at a sufficiently high specific activity with [^{14}C]-galactose. Conversely, we could obtain good incorporation into the more heavily glycosylated IgM that is synthesized and secreted by J[μ1] cells (Sitia *et al.*, 1990). Moreover, using these cells experimental conditions can be selected in which the requirements for H-L assembly and polymerization can be identified separately.

In agreement with the concept that IgM polymerization occurs before the transGolgi (Sitia *et al.*, 1990; Brewer *et al.*, 1994), only the polymeric form of IgM is labeled in the lysates of J[μ1] cells after a 15-min pulse with [^{14}C]-galactose (Figure 7A, lane 1). When cells are chased for 20 min, more than one-half of the radiolabeled IgM is secreted, mostly in the form of polymers (Figure 7A, lane 3). The continuous presence of 2 mM DTT during a 20-min chase inhibits secretion only slightly. In SDS-PAGE secreted material appears to be constituted by a few intermediate oligomers, monomers, hemimers, and also some molecules migrating as free μ chains (Figure 7A, lane 5). Because DTT was present in the chase medium, these forms could have been generated extracellularly. However, μ2L2 and μL complexes, which are normally retained intracellularly in the absence of reducing agents (Alberini *et al.*, 1990; Sitia *et al.*, 1990), are detected in the supernatants also when cells are exposed to DTT for the first 10 min of chase, washed, and cultured for further 10 min without DTT (Figure 7A, lane 7). As a control, purified radioactive IgM polymers are not reduced when they are coincubated for 10 min with unlabeled J[μ1] cells treated with DTT and washed as above (Figure 7B). Hence, galactosylated monomers and hemimers are not generated by extracellular reduction of secreted polymers. Are they secreted as such or as noncovalent polymers? Mild reduction is known to cause depolymerization of purified IgM in vitro (DellaCorte and Parkhouse, 1973). To verify whether this is true also for galactose-labeled IgM from the transGolgi, the lysates of J[μ1] cells, chased in the presence of DTT as above, were fractionated by nondenaturing sucrose gradients. Individual fractions were then precipitated by NP-sepharose and analyzed by nonreducing SDS-PAGE (Figure 8A). In cells chased for 10 min with DTT and 10 min without reducing agent, μ2L2 and μL molecules migrate in fractions well separated from those containing polymers (Figure 8, lanes 1–3 and 6, respectively), indicating that they are not part of noncovalent polymers. In addition, the stoichiometry of μ2L2 and μL varies in individual fractions (Figure 8A, lanes 1–3), indicating that some μ2L2 complexes are not only reduced but actually dissociated into μL hemimers by DTT. Only traces of noncovalent polymers are detected (Figure 8A, lane 12) after 20 min of chase in the continuous presence of DTT. The fractions corresponding to the monomer peak (Figure 8A, lanes 8–10) contain molecules migrating in SDS-PAGE as μL and μ. However, the latter are likely to derive from noncovalent μL (or μ2L2) complexes, because they are reactive with NP-sepharose.

To further determine to what extent DTT induces H-L disassembly of galactosylated IgM from the trans-Golgi, the lysates and supernatants of cells chased for 20 min in the presence of the reducing agent were precipitated by anti-μ or anti-λ. Our anti-λ antibodies are less efficient than anti-μ in precipitating μL and higher order assemblies (Figure 8B, compare lanes 2 and 4). Taking this into account, it appears that anti-λ precipitates most if not all the extracellular μ-chain associated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radioactivity, including the band migrating as free μ chains. The latter must hence derive from noncovalently associated μ-L complexes. In contrast, in the cell lysates the μ/μL ratios are higher in anti-μ than in anti-λ (Figure 8B, compare lanes 1 and 3), indicating that there is some disassembly of galactose-labeled IgM, which results in transport incompetence of free μ chains. However, the extent of DTT-induced μ-L disassembly is much lower for galactose-labeled than for newly synthesized molecules. Taken together, these results indicate that IgM labeled in the transGolgi are partially resistant to DTT-mediated reduction and that this event is not accompanied by a loss of transport competence, which is evident only in the case of the few fully disassembled μ chains. As a result, galactose-labeled unpolymerized IgM appear to be transported through the transGolgi network to the extracellular space despite the presence of an exposed μ tailpiece, suggesting that Cys575-mediated quality control is not operational in or beyond the transGolgi.

After 1 h of Chase, DTT no Longer Blocks Secretion of IgM

We then compared the effects of DTT on the same pool of IgM molecules as they migrate along the exocytic pathway (Figure 9) by the experiment outlined in Figure 9C. J[μ1] cells were pulsed with [^{35}S]-methionine and cysteine and chased in the presence of DTT for 20 or 60 min either immediately after the pulse (Figure 9, grey arrow) or after 1 h of chase in the absence of the reducing agent (Figure 9, black arrow). Samples were immunoprecipitated with anti-μ antibodies or NP-sepharose and analyzed under reducing or nonreducing conditions (Figure 9, A and B).

As in the case of IgG, the presence of DTT in the first hour of chase inhibits IgM secretion. In contrast, if DTT is added after 1 h of chase at 37°C, when intracellular (Figure 9A, lane 1) and extracellular polymers are detected in abundance, secretion is only partially inhibited (Figure 9A, compare lanes 10 and 11). In nonreducing SDS gels the main forms accumulating extracellularly in the presence of DTT (Figure 9A, lanes 7 and 9) are μL and μ. Also in this case, however, the latter derive from noncovalently associated μL or higher order complexes; they are infact quantitatively precipitated by NP-sepharose (Figure 9B, compare the intensity of the μ chain bands in lanes 2 and 5).

It is noteworthy that after 60 min of chase with DTT, two forms of μ chains are detected extracellularly under both nonreducing and reducing conditions (see Figure 9A, lanes 9 and 11, and Figure 9B, lanes 2 and 5). As determined by endoglycosidase H sensitivity assays (Figure 9B, lanes 3 and 4), the two molecules represent differently glycosylated μ isoforms. The more rapidly migrating band, the only one detectable after 20 min of chase (see Figure 9A, lane 7), comigrates with μ chains secreted in the absence of reducing agents (compare Figure 9B, lanes 1 and 2, or Figure 9A, lanes 10 and 11). The slower form, indicated here as μ*, comigrates instead with μ chains that are transported through the Golgi and secreted as μ2L2 monomers (Davis *et al.*, 1989a; Sitia *et al.*, 1990; Cals, Guenzi, Simmen, Sparvoli, and Sitia, unpublished data). This latter form originates from the processing of the C-terminal glycosylation site, which is otherwise inaccessible to the Golgi glycosidases and glycosyltransferases in polymeric IgM (Cals, Guenzi, Simmen, Sparvoli, and Sitia, unpublished data). Thus, the presence of μ* confirms that in the presence of reducing agents, including DTT when added in the correct time frame, nonpolymeric IgM can traverse the Golgi apparatus as such and are not generated extracellularly.

DISCUSSION

Three main conclusions can be drawn from our studies: 1) DTT, but not 2ME, induces reduction and partial disassembly of intracellular Igs; 2) Ig folding and assembly need not occur cotranslationally; and 3) thiol-mediated quality control, so efficient in the ER, becomes of little if any importance in or beyond the Golgi.

Dissecting the Sequential Quality Control of Igs by the Use of Reducing Agents

Although the addition of reducing agents to living cells might affect many metabolic pathways, their effects on the transport of membrane or secretory molecules along the exocytic pathway appear to be specific for individual cargo proteins. Thus, 2ME does not induce secretion of proteins that are retained by mechanisms other than disulphide interchange (Alberini *et al.*, 1990), and DTT does not block the transport of proteins devoid of SS bonds (Chanat *et al.*, 1993; Lodish and Kong, 1993). Furthermore, in our myeloma transfectants secretion of free L chains is inhibited by DTT to a lesser extent than that of H chains (Figure 3), confirming that the reducing agent does not block ER-Golgi vesicular traffic altogether.

Figure 6. Retention of IgG2b-μtpCys in a pre-Golgi compartment is reversed by 2ME but not by DTT. J[γ2b-μtpCys] cells were cultured for 1 h with 4 mM 2ME (b, e, h, and j), 2 mM DTT (c and f), or in plain culture medium (a, d, g, and i) before formaldehyde fixation and detergent permeabilization. Sets of slides were costained with fluoresceinated anti-γ (a–c) and with anti-BiP followed by rhodaminated anti rat Ig (d–f), or with rhodaminated anti-γ (g and h) and fluoresceinated ricin-A (i and j). Arrows in h and j point to the colocalization of the anti-γ and ricin-A staining in the Golgi area after 2ME treatment, from which BiP is excluded (arrows in b and e). Note that in most untreated cells the Golgi area is weakly or not stained by anti-γ (see arrows in g and i). Bar = 8 μm.

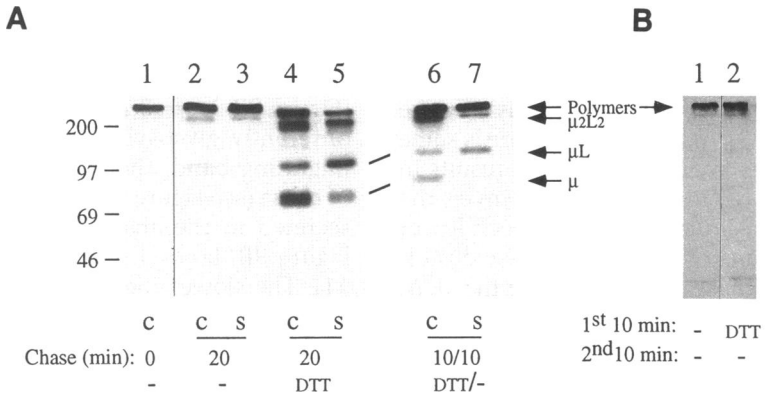

Figure 7. DTT does not inhibit the exit of galactose-labeled IgM from the transGolgi. (A) Galactose-labeled IgM assembly intermediates accumulate extracellularly in the presence or absence of DTT. J[μ1] cells, which produce and secrete wild-type IgM, were pulsed for 15 min with [^{14}C]-galactose (lane 1) and chased for 20 min with (lanes 4 and 5) or without (lanes 2 and 3) 2 mM DTT in a medium containing excess cold galactose and glucose. An aliquot of cells was chased for 10 min in the presence of DTT, washed, and cultured for further 10 min without reducing agents (lanes 6 and 7). IgM was immunoprecipitated from cell lysates (c) and supernatants (s) and resolved on SDS-PAGE (10%) under nonreducing conditions. (B) Reduction of IgM does not occur extracellularly. Unlabeled J[μ1] cells were treated for 10 min with (lane 2) or without (lane 1) 2 mM DTT, washed, and cultured for further 10 min without the reducing agent. Immunopurified [^{35}S]-labeled polymeric IgM were added for the last 10 min of culture. Aliquots of the supernatants were then resolved on a 3–14% gradient SDS-PAGE under nonreducing conditions.

The observation that DTT, but not 2ME, induces reduction and partial dissociation of newly formed H2L2 assemblies (Figures 2 and 3) explains the different effects of the two reducing agents on Ig secretion. Dithiols are known to be more effective than monothiols in reducing intra- and intermolecular disulphides (Gilbert, 1990). Leaving the H2L2 structures undisturbed, 2ME displays only positive effects on the secretion of intermediates that are retained by disulphide interchange reactions involving Cys575 (Alberini *et al.*, 1990; Fra *et al.* 1993; Guenzi *et al.*, 1994). In contrast, the H2L2 disassembly induced by DTT may reexpose H chains to other quality control mechanisms in charge of verifying H-L assembly or HL dimerization (Bole *et al.*, 1986; Hendershot *et al.*, 1987; Sitia *et al.*, 1990) and independent from disulphide interchange reactions. Yet another level of quality control, that corresponding to folding of individual subunits, becomes evident in the case of L chains. The latter can be secreted also in the monomeric form (Knittler and Haas, 1992), and their retention correlates primarily with unfolding, as monitored in our experiments by reduction of the intrachain SS bonds (Figures 3 and 4). The secretion of oxidized L chains in the presence of DTT, at concentrations that prevent the transport of IgG, in all likelihood reflects the high propensity to intrachain disulfide bond formation that is evident during Ig biogenesis (Bergman and Kuehl, 1979a,b). If reduction is not accompanied by extensive unfolding, the local concentration of the relevant cysteines might be high enough to drive the formation of oxidized molecules that can escape the quality control system.

Retention and degradation are tightly coupled events in the quality control of newly synthesized proteins (Bonifacino and Lippincott-Schwartz, 1991; Knittler and Haas, 1992; Fra *et al.*, 1993; Young *et al.*, 1993). Whereas in the absence of reducing agents γ2b chains are rather stable even when expressed in cells lacking L chains (Fra *et al.*, 1993), chimeric Igs are rapidly degraded in the presence of reducing agents, DTT in particular. The reversibility of the DTT effects suggests that degradation is not the cause but rather the consequence of the secretory block. The disassembly and unfolding induced by DTT are likely to facilitate the activity of the ER proteolytic machinery.

Posttranslational Folding and Assembly of Ig Molecules

The classical experiments of Bergman and Kuehl (1979a,b) demonstrated that folding and assembly of nascent Ig molecules occur cotranslationally. However, the results of Figures 3 and 4 show that, as in the case of other proteins (Braakman *et al.*, 1992a,b; Kaji and Lodish, 1993a,b), these events can also take place posttranslationally. The possibility of assembling, disassembling, and reassembling proteins, which we observation in our experiments with DTT, is probably a normal feature of the maturation of oligomeric complexes in the ER. This dynamic quality control can be viewed as a dual failsafe mechanism that on the one hand prevents secretion of misassembled molecules and on the other contributes to their correct maturation by keeping them in an optimal environment (Helenius *et al.*, 1992).

Localizing Quality Control Along the Exocytic Pathway

The role of disulphide bonds in folding, assembly, and oligomerization varies from protein to protein. For in-

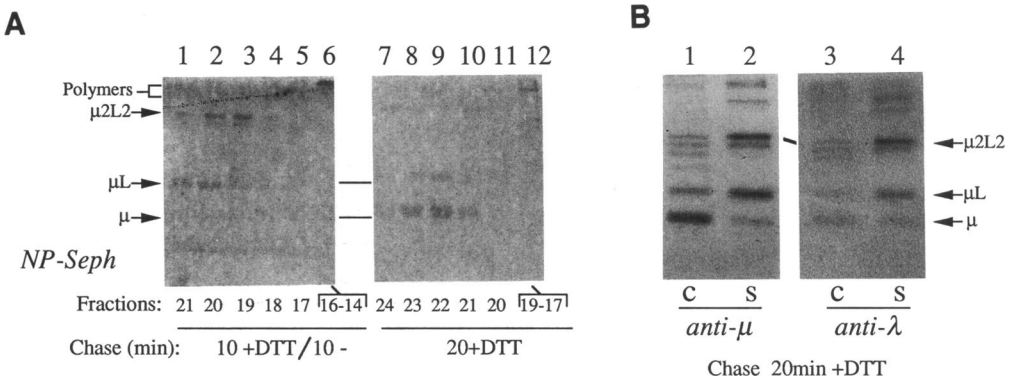

Figure 8. DTT induces partial disassembly of polymeric IgM from the transGolgi. (A) DTT induces depolymerization of galactose-labeled IgM. J[μ1] cells were pulsed with galactose and chased with DTT for 20 min (lanes 7–12) or 10 min followed by further 10 min without reducing agents (lanes 1–6) as in Figure 7A. Cell lysates were fractionated by continuous sucrose gradients (5–20%), and fractions were immunoprecipitated by NP-sepharose and resolved by nonreducing SDS-PAGE. In both panels the lanes on the right derive from the pool of three fractions corresponding to the polymer peak. See MATERIALS AND METHODS for further details. (B) Secretion of galactosylated noncovalent μ-L complexes in the presence of DTT. The lysates (c) and supernatants (s) of galactosylated J[μ1] cells, chased for 20 min with DTT, were immunoprecipitated by anti-μ (lanes 1 and 2) or anti-λ (lanes 3 and 4) and resolved on a 3–14% gradient SDS-PAGE under nonreducing conditions.

stance, whereas IgM polymerization largely depends on the formation of the interheavy chain SS bonds (DellaCorte and Parkhouse, 1973; Davis *et al.*, 1989b; Sitia *et al.*, 1990), the H-L interchain disulphide is not essential for H2L2 assembly and stability (Kerr, 1990). In addition, neither H-L disassembly nor unfolding of H and L chains are induced by reducing agents in vitro, unless denaturing agents are added (Sears *et al.*, 1975; Valetti, Fagioli, and Sitia, unpublished data).

Why then does DTT induce H2L2 disassembly intracellularly? In vivo, the DTT-induced disassembly is more efficient for newly made proteins than for molecules that have been allowed to reach the Golgi, suggesting the existence of additional mechanisms specifically located in the ER or a related compartment, probably involving ER resident chaperone molecules (Kaji and Lodish, 1993b; Tatu *et al.*, 1993). Perhaps the alterations in the redox potential induced by DTT modulate also the activity of ER chaperones so as to preferentially catalyze unfolding and disassembly. Transport incompetence may result from rapid kinetic reactions rather than from stable interactions with ER resident proteins. In fact, the efficacy of the secretory block is often in contrast with the partial reduction of inter- or intrachain disulphides. This might also reflect the existence of transport incompetent intermediates with subtle differences in conformation, not detectable by our gel or precipitation assays, as described in the case of retinol-binding protein (Kaji and Lodish, 1993a).

Are the quality control functions limited to the ER, or are they distributed along the entire exocytic pathway? Two features make Igs a suitable model to tackle these questions: 1) unlike in the case of viral proteins (de Silva *et al.*, 1990; Tatu *et al.*, 1993), the Ig interchain SS bonds can be reduced by DTT along the entire secretory apparatus and 2) folding, assembly, and polymerization can be dissected independently.

The failure of DTT to block the exit of Ig molecules that have reached the Golgi correlates with the incapability of dissociating HL assemblies, despite the fact that the interchain SS bonds are reduced. Thus, although the unequal distribution of galactose-labeled μ and μL in cell lysates and supernatants (Figures 7 and 8) might reflect the presence of active BiP molecules also in the distal sections of the secretory apparatus (Hammond and Helenius, 1994), the first steps of Ig quality control (folding and H-L assembly) seem to be primarily localized in a pre-Golgi compartment. Several lines of evidence indicate that this is the case also for the thiol-mediated quality control that determines the fate of unpolymerized IgM and IgA during B cell development (Fra *et al.*, 1993; Guenzi *et al.*, 1994). First, galactosylated IgM monomers and hemimers generated in the transGolgi by treatment with DTT can be secreted also in the absence of reducing agents (Figure 7). Second, 2ME is required for the mobilization of thiol-exposing IgG2b-μtpCys from the ER to the Golgi but not for the subsequent steps along the exocytic pathway (Figure 5). Further insights come from analyzing the state of the μ chain carbohydrates. The μ chain C-terminal glycosylation site (Asn563) is normally found in the high mannose configuration in secreted IgM polymers (Brenckle and Kornfeld, 1980; Anderson *et al.*, 1985; Wormald *et al.*, 1991) but becomes processed when μ chains are secreted as μ2L2 monomers (Davis *et al.*, 1989a; Sitia *et al.*, 1990) because of differential accessibility to the Golgi glycosidases and glycosyltransferases in monomeric and polymeric IgM (Cals, Guenzi, Simmen, Sparvoli, and Sitia, unpublished data). These findings confirm that IgM polymerization precedes the arrival to the Golgi. The presence of μ* molecules in the supernatants of DTT-treated cells (Figure 9) implies that monomers are generated intracellularly and are exposed to all the crucial sugar-modifying enzymes. The delayed

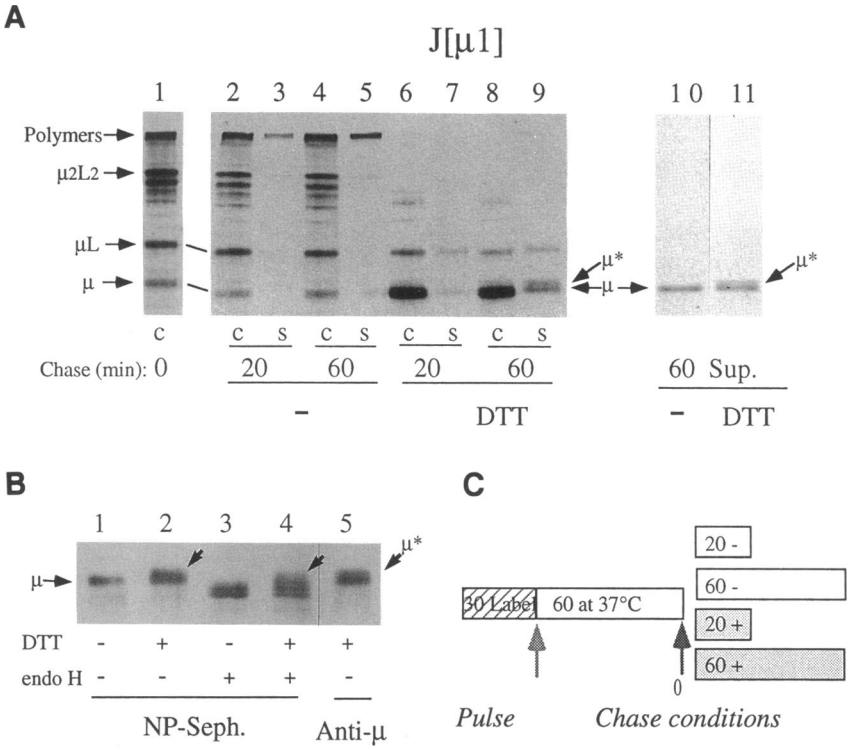

Figure 9. After 1 h of chase, some methionine-labeled IgM become resistant to the DTT-induced secretory block. As outlined in C, J[μ1] cells were pulsed for 30 min with [^{35}S]-methionine and cysteine, chased for 1 h in complete medium (lane 1 of A, considered time 0 of chase), washed once, and chased for 20 or 60 min in the presence or absence of 2 mM DTT as indicated. (A) The anti-μ immunoprecipitates from cell lysates (c) and supernatants (s) were resolved under nonreducing (lanes 1–9) or reducing (lanes 10 and 11) conditions by SDS-PAGE (6 and 10%, respectively). The two gels were aligned based on the migration of μ chains. Polymers, μ2L2, μL, and free μ chains are indicated by arrows on the left hand margin. Hyperglycosylated μ chains present in the supernatants of cells chased for 60 min in the presence of DTT (lanes 9 and 11) are indicated as μ* on the right hand margin (see below). (B) Aliquots of the supernatants harvested after 60 min of chase with (lanes 2, 4, and 5) or without (lanes 1 and 3) DTT were precipitated with NP-sepharose (lanes 1–4) or anti-μ (lane 5) and resolved on reducing gels (10%) after overnight incubation with (lanes 3 and 4) or without endoglycosidase H. Arrows on lanes 2 and 5 point to μ* chains, detected in the supernatants of DTT-treated cells. As indicated by the appearance of an endoglycosidase H resistant form (see arrow on lane 4), μ* molecules represent a more glycosylated isoform of μ chains. The μ chain doublet present also in lane 3 may reflect heterogeneous glycan processing (Anderson *et al.*, 1985; Sitia *et al.*, 1987), possibly at the Asn402 site (Sun *et al.*, 1991). (C) Outline of the experimental scheme employed.

appearance of μ* chains in the supernatants (>20 min after DTT addition) is consistent with the assumption that DTT can generate IgM monomers and hemimers in the cisGolgi, where the mannosidases reside (Nilsson *et al.*, 1993), and does not prevent their further transport along the secretory apparatus.

In conclusion, secretory Igs are subject to little quality control once they have reached the Golgi. However, several proteins have been reported to oligomerize in post-ER compartments (Rotundo, 1984; Colley and Baezinger, 1987; Wagner, 1990; Bonifacino and Lippincott-Schwartz, 1991; Jascur *et al.*, 1991; Huovila *et al.*, 1992; Musil and Goodenough, 1993). It will be of interest to determine whether these molecules are subject to distally located quality control systems.

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