Quality control in protein biogenesis: thiol-mediated retention monitors the redox state of proteins in the endoplasmic reticulum

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SUMMARY

There is accumulating evidence that proteins can be retained in the endoplasmic reticulum by a mechanism that is believed to monitor the oxidation status of one or more cysteines in their sequences. For example, a single cysteine residue critical for retention of secretory IgM assembly intermediates has been mapped to the C-terminal cysteine, Cys575, of the secretory μ chain. Little is known concerning the mechanism responsible for this system of quality control, which has been termed thiol-mediated retention. In particular, it is not known if the mechanism monitors the redox state of the important cysteine residue in the secretory μ protein itself or within the context of higher-order IgM complexes. To address this question, we evaluated the fidelity of retention of secretory IgM and determined the redox status of cysteines in secretory μ proteins in polymers and polymer intermediates at various stages of maturation. We demonstrate that all secreting B cells and B cell lines secrete assembly intermediates in addition to completed, covalent pentameric and hexameric IgM polymers. A fraction of assembly intermediates exit the endoplasmic reticulum as individual components, mature through the Golgi without undergoing further assembly, and most, if not all, are secreted. While the majority of IgM assembly intermediates have exposed thiols and are contained within the endoplasmic reticulum where they can be utilized for oligomerization, maturing assembly intermediates found in the Golgi and extracellular space are completely oxidized. Thus, while the retention of unpolymerized IgM is highly efficient, the retention system lacks the ability to distinguish fully oxidized assembly intermediates from fully oxidized completed polymers. The molecular mechanisms that may contribute to this aspect of IgM biogenesis and their implications for the concept of thiol-mediated retention are discussed.

Key words: Endoplasmic reticulum, Thiol-mediated retention, Quality control, Secretion, IgM

INTRODUCTION

The transport of multi-subunit protein complexes requires that properly folded molecules undergo correct and complete assembly into the proper quaternary structure (Gething et al., 1986; Kreis and Lodish, 1986; Hurtley and Helenius, 1989; Hammond and Helenius, 1995). One level of quality control is mediated by resident endoplasmic reticulum (ER) chaperones, such as BiP/GRP78 (Haas, 1994) and calnexin (Bergeron et al., 1994), which bind and retain incompletely or improperly assembled protein complexes, thus preventing their transport through the secretory pathway. For example, an immunoglobulin (Ig) heavy (H) chain is stably bound to BiP/GRP78 until the H chain successfully assembles with an Ig light (L) chain to form a secretion competent complex (Hendershot, 1990). Additional, perhaps distinct, mechanisms of quality control also appear to operate in the secretory pathway. This is particularly evident in the biogenesis of polymeric secretory IgM. Completely polymerized IgM pentamers (μ2L2)5 or hexamers (μ2L2)6 are secreted, while the IgM assembly intermediates, including the most abundant μL half-monomers and μ2L2 monomers are efficiently retained within the ER (Davis and Shulman, 1989; Sitia et al., 1990; Brewer et al., 1994a,b). This represents a critical factor in IgM biogenesis since two important immunologic roles of this antibody isotype, complement activation and transcytosis across epithelial cells into exocrine secretions, are dependent on its final polymeric structure (Borsos and Rapp, 1965; Mostov and Blobel, 1983).

Like the quality control mechanism responsible for the retention of IgM assembly intermediates, IgM polymerization has been localized to an early compartment of the secretory pathway, most probably the ER (Tartakoff and Vasalli, 1979; Brewer et al., 1994b; Bornemann et al., 1995). Polymerization is a dynamic process in which non-covalent interactions between assembling subunits precede covalent completion of pentameric and hexameric IgM polymers (Brewer et al., 1994b). The C-terminal cysteine of the secretory μ (μs) chain (Cys575), contained within the 20 amino acid sequence of the μ secretory tailpiece, is essential for the covalent polymerization of μL and/or μ2L2 subunits into higher-order structures.
MATERIALS AND METHODS

Mice
Mice were purchased from Jackson Laboratories (Bar Harbor, ME). B10.A mice were used for passaging the CH12 cells. Serum and spleen cells were obtained from C57BL/6 mice, while serum from Trypanosoma brucei infected BALB/c mice was kindly provided by A. Balber (Duke Medical Center, Durham, NC).

Cells and cell culture
The IgM-secreting hybridomas S12M1 and BrM8 (Conger et al., 1989) and Sp6HL (kindly provided by M. J. Shulman, University of Toronto; Köhler et al., 1982) and the inducible B cell lymphoma, CH12 (Ovnic and Corley, 1987), have been described previously. CH12 cells were stimulated with lipopolysaccharide (LPS) (Escherichia coli 055:B5; Difco Laboratories, Detroit, MI) at 50 μg/ml or with IL-5 (Genzyme, Cambridge, MA) at 25 U/ml for 48 hours to induce high-rate antibody secretion (Randall et al., 1992). Spleen cells were prepared from 4-6 week old C57BL/6 mice as described (Kuhara et al., 1985) and cultured at 1×10^6 cells/ml in the presence of LPS at 10 μg/ml for 5 days. Cells were cultured in DMEM with 10% FBS, 50 μM 2-ME, and supplemented as previously described (Ovnic and Corley, 1987).

Biosynthetic labeling, immunoprecipitation and endo H treatment
Cells were washed in balanced salt solution (BSS), resuspended at 1-5×10^6 cells/ml in methionine- and cysteine-free DMEM (ICN, Costa Mesa, CA) for 20 minutes, and metabolically labeled in the same medium with 35-100 μCi/ml of Tran35S-label (ICN) or Expre35S35S-label (Dupont NEN, Boston, MA) for 2 hours. For labeling galactose residues, cells were cultured at 5×10^6 cells/ml for 2 hours in glucose-free DMEM (Gibco Laboratories, Grand Island, NY) containing 150 μCi/ml of [6-3H]D-galactose (ICN). All labeling medium was supplemented as regular medium except for containing 10% dialyzed FBS. Labeled cells were washed twice in ice-cold BSS containing 20 mM N-ethylmaleimide (NEM, Sigma, St Louis, MO) and frozen until use.

To control for the possible effects of the 2-ME in the 35S-labels, labeling was performed under conditions in which the final concentration of 2-ME was either 50 μM or at concentrations well below those determined to alter the profile of secreted intermediates (Alberini et al., 1990; and our unpublished data), at a maximum concentration of 200 μM. In addition, metabolic labeling with 3H-galactose was compared in the presence (50 μM) and absence of 2-ME. No differences in the amount or distribution of secreted intermediates was detected.

Cell lysates were prepared in 1% NP-40 exactly as described (Brewer et al., 1994b). Before specific immunoprecipitation, lysates and supernatant samples were precleared with normal rabbit serum (NRS) together with 25% Staphylococcus aureus crude cell suspension (Sigma) or Protein A-Sepharose (Sigma). IgM was then affinity-purified by immunoprecipitation with a rabbit anti-mouse μ chain specific antibody (ICN) and Protein A-Sepharose. A rabbit anti-mouse J chain antisera, a gift from R.M.E. Parkhouse (Kaji and Parkhouse, 1974), was used to immunoprecipitate J chain. Precipitated material was eluted from the beads with the appropriate electrophoresis sample buffer or eluted for treatment with endoglycosidase H (endo H, Boehringer, Indianapolis, IN) exactly as described (Brewer et al., 1994b).

Analysis of IgM
Western blotting was performed using a 125I-labeled goat anti-mouse μ chain antibody (Randall et al., 1992). The IgM concentration of supernatant samples and of sucrose gradient fractions was determined by quantitative ELISA (Randall et al., 1990).

Gel electrophoresis
Non-reducing 4% PAGE under non-denaturing (native) conditions and standard reducing 10.5% SDS-PAGE were carried out as described (Randall et al., 1990). Electrophoresis of IgM under non-reducing, denaturing conditions by agarose/SDS-PAGE was performed using gels containing 2.5% polyacrylamide and 0.5% agarose exactly as described (Brewer et al., 1994b). Two-dimensional (2-D) non-reducing/reducing gels using agarose/SDS-PAGE tube gels in the first dimension and reducing SDS-PAGE in the second dimension were performed as described (Brewer et al., 1994b). Prestained molecular size markers (SDS-7B, Sigma) were used on all reducing SDS-PAGE. Gels were prepared for western blotting or autoradiography as described (Brewer et al., 1994b). Portions of gels...
corresponding to bands of interest were quantitated directly by excising them from the dried gel, solubilizing in Aquasol-2 (Dupont), and analyzing by scintillation spectroscopy.

Sucrose gradient fractionation
Cell lysates and culture supernatants were fractionated on 11-step 5%-20% sucrose gradients containing 1% NP-40 lysis buffer (Brewer et al., 1994b). Prior to pouring gradients, gradient tubes were pre-coated overnight with phosphate buffered saline (PBS) containing 10 mg/ml BSA. Gradients were manually tapped from the bottom, and fractions of approximately 240 or 480 μl were collected. For unlabeled samples, IgM content of individual fractions was determined by quantitative ELISA and analyzed by non-reducing agarose/SDS-PAGE and western blotting. For labeled samples, individual fractions were screened in duplicate for IgM by scintillation spectroscopy following immunoprecipitation, and then further analyzed either by non-reducing agarose/SDS-PAGE or by 2-D.

Radioalkylation
Cells were washed in warm DMEM and then resuspended at 5x10^5 cells/ml in warm DMEM supplemented as before except that the medium contained no 2-ME and the FBS had been prealkylated with NEM. Pretreatment of FBS with NEM was carried out to block NEM-sensitive sites on serum proteins that might compete with secreted IgM for alkylation. FBS was treated with 100 mM NEM for 1 hour and then dialyzed against PBS. ELISA analysis revealed that IgM secretion was not affected by short-term culture in the presence of alkylated FBS. Cells were cultured for 4 hours and then centrifuged, and a supernatant sample was collected. The cells were washed twice with ice-cold PBS, and 1x10^5 cells were lysed on ice for 30 minutes in 200 μl of NP-40 lysis buffer as described (Brewer et al., 1994b) containing 100 μCi of [3H]NEM. A 200 μl sample of culture supernatant was also treated with 100 μCi of [3H]NEM. A lysate and culture supernatant sample from 2.5x10^6 cells was prepared in parallel without [3H]NEM. Immunoprecipitation, endo H treatment, and reducing SDS-PAGE was performed as described above.

RESULTS
Secretion of incompletely polymerized IgM
To examine the extent to which IgM assembly intermediates are secreted, we first analyzed the polymeric forms of IgM secreted from a panel of B cell lines. IgM was resolved on non-reducing, denaturing gels and examined by western blotting to identify covalent polymers and assembly intermediates (Brewer et al., 1994b). The predominant forms of IgM present in each sample were the completed pentamers and/or hexamers (Fig. 1A, left). However, additional μ-containing proteins were present in lower abundance as revealed by a longer exposure of the blot (Fig. 1A, right). The most prevalent of these migrated to a position consistent with its identity as the μ2 L2 monomer, a complex of approximately 200 kDa containing both μ and L chains (Brewer et al., 1994b). A variety of other higher-order IgM assembly intermediates were also present in each sample. Interestingly, for all cell lines examined the whole-step intermediates (monomer, dimer, trimer, and tetramer) were secreted in greater abundance than the half-step intermediates (half-monomer, monomer-μ, etc.). These results demonstrate that incompletely polymerized forms of IgM, primarily the IgM monomer, are part of the normal distribution of IgM secreted from cultured B cell lines.

We also analyzed the IgM secreted by normal B cells both

Fig. 1. The polymeric complexity of secreted IgM.
IgM was detected by western blotting using a labeled anti-μ probe. Completed polymers, both pentamers and hexamers, are denoted by ‘P’, and monomers by ‘M’.
(A) Culture supernatants of LPS-stimulated CH12 cells and three IgM-secreting B cell hybridomas (BrM8, Sp6, and S12M1) were analyzed by non-reducing agarose/SDS-PAGE. A short exposure (left panel) and a 4-fold longer exposure (right panel) of the blot are shown. (B) Serum was obtained from two individual mice (1 = female; 2 = male) and the spleen cells from these same mice were then stimulated with LPS. The IgM concentration of the serum and culture supernatants was determined by quantitative ELISA and equivalent amounts of IgM were then examined by agarose/SDS-PAGE. (C) An equivalent amount of serum from a normal mouse and a T. brucei infected mouse was examined by agarose/SDS-PAGE. (D) CH12 cells were cultured in medium alone, with LPS, or with IL-5. Equal samples of culture supernatant were analyzed by non-denaturing (native) PAGE. A short exposure (left panel) and a 10-fold longer exposure (right panel) of the blot are shown. Note that in each experiment, A-D, secreted IgM was predominantly in the completed polymer form and, in much lesser abundance, in various forms of incomplete polymers, primarily the monomer.
in vivo and in vitro. Spleen cells were stimulated with LPS and the culture supernatants were analyzed together with serum samples from the same mice. As expected, the IgM secreted in vitro by normal B cells as well as the IgM present in the serum of normal mice consisted primarily of completed IgM polymers (Fig. 1B). However, other forms of IgM, primarily whole-step intermediates, were observed in these samples and the most abundant of these was monomeric IgM. The secretion of incomplete IgM polymers is, therefore, not an artifact of the most abundant forms of IgM were detected by western blotting. Both LPS and IL-5 stimulated CH12 cells to secrete predominantly completed IgM polymers (Fig. 1A). Cells were metabolically labeled with [35S]methionine and -cysteine. J-containing proteins were immunoprecipitated from cell lysates (left panel) and culture supernatant (right panel) and treated with endo H. Proteins were separated in the first dimension by non-reducing agarose/SDS-PAGE and then resolved in the second dimension by reducing SDS-PAGE. Maturing, endo H-resistant intracellular assembly intermediates and secreted intermediates are marked with asterisks. Completed polymers, including pentamers and hexamers, are marked with brackets. Note that the profile of maturing (endo H resistant) intracellular IgM assembly intermediates is identical to the profile of secreted IgM intermediates. An endo H treated anti-J chain immunoprecipitate from labeled cells serves as a marker (M) for J chain identification.

Maturing, endo H resistant intracellular IgM is destined for secretion

We next asked if we could detect assembly intermediates intracellularly as they mature along the secretory pathway. The profile of maturing endo H resistant intracellular IgM was compared to the profile of secreted IgM from the hybridoma, BrM8, a cell line which secretes readily detectable amounts of assembly intermediates (Fig. 1A). Cells were metabolically labeled with [35S]methionine and -cysteine, intracellular and secreted IgM was immunoprecipitated and treated with endo H. The proteins were resolved by 2-D (non-reducing/reducing) gel analysis (Fig. 2). s chains migrate more slowly in the second dimension than the immature forms (Fig. 1A). Cells were metabolically labeled with [35S]methionine and -cysteine, intracellular and secreted IgM was immunoprecipitated and treated with endo H. The proteins were resolved by 2-D (non-reducing/reducing) gel analysis (Fig. 2). s chains, a fraction of intracellular s chains are resistant to endo H cleavage, and these represent primarily the s chains in covalent polymers that have matured through the mid-Golgi (Anderson et al., 1985; Davis et al., 1989; Cals et al., 1996). While treatment of intracellular secretory IgM with endo H results in the cleavage of all five N-linked oligosaccharides, four of which are processed into endo H resistant forms upon transport through the mid-Golgi (Anderson et al., 1985; Davis et al., 1989; Cals et al., 1996). The faint band visible just below the top and are predominantly endo H sensitive; the mature endo H resistant s chains migrate more slowly in the second dimension than the immature forms (Fig. 2). The k light chain, represented by the intense horizontal line nearer the bottom of the gel, lacks N-linked glycans and is not affected by endo H treatment. The faint band visible just below k is J chain (Brewer et al., 1994b). As shown in Fig. 2, such an analysis reveals a profile of endo H resistant intracellular completed polymers, monomers, and other assembly intermediates (Fig. 2, left panel) which is strikingly similar to the profile of
secreted completed polymers and assembly intermediates (Fig. 2, right panel). The predominant form of mature, endo H resistant intracellular IgM and secreted IgM was, as expected, the completed polymers (marked with brackets). The next most abundant form of mature intracellular IgM corresponded to monomeric IgM (Fig. 2, left panel), consistent with the monomer being the most prevalent of the secreted assembly intermediates (Fig. 2, right panel). In addition to mature monomers, there were three other clearly resolved endo H resistant higher-order assembly intermediates present intracellularly and in secreted material. These complexes were resolved at positions between monomers and completed polymers and migrated as expected for IgM dimers, trimers, and tetramers. We attempted to use phosphorimaging analysis to compare the relative proportions of intracellular maturing and secreted intermediates. However, the overlap in the signals, especially between intracellular endo H sensitive and endo H resistant intermediates, made exact quantitation impossible. Nevertheless, there is a clear correlation between the polymeric complexity of maturing intracellular IgM and that of secreted IgM, indicating that most, if not all, of the IgM assembly intermediates that exit the ER are secreted.

Non-covalently assembled polymers do not mature along the secretory pathway

We next focused on understanding how intracellular IgM assembly intermediates are transported through the secretory pathway. We reasoned that non-covalently assembled IgM pentamers or hexamers might meet quality control standards and be allowed to exit the ER, thereby providing a vehicle for the transport of assembly intermediates through the remainder of the secretory pathway. Alternatively, a fraction of intermediates may exit the ER as individual components, mature through the secretory pathway, and be secreted with no dependence on transport via polymer structures.

To distinguish between these possibilities, we first determined whether non-covalently assembled IgM polymers are ever secreted. Cell lysates and culture supernatant from BrM8 cells were fractionated on sucrose density gradients to separate completed polymers from smaller IgM oligomers under non-denaturing conditions (Fig. 3, upper panels), and the covalent complexity of the IgM present in the gradient fractions was assessed on denaturing/non-reducing gels (Fig. 3, lower panels). As shown in the left panels, the majority of intracellular IgM was contained in a broad peak at the upper end of the gradient (fractions 28-37) and consisted of monomers and half-monomers. A small pool of completed polymers was identified in a peak at the lower end of the gradient (Fig. 3, upper left). Various assembly intermediates, including the low-abundance half-step intermediates (Brewer et al., 1994b), were found between these two predominant peaks. An analysis of the fractions containing intracellular polymers (Fig. 3, lower left, fractions 10-19) on denaturing, non-reducing gels showed that they contained both covalently and non-covalently assembled polymers, since a variety of assembly intermediates, including monomers and half-monomers, were detected in these samples. The other assembly intermediates, including the high-abundance monomers and half-monomers were efficiently separated on the gradient (Fig. 3, lower left, fractions 22-37). Thus, non-covalently assembled polymers are present intracellularly. This contrasted with secreted IgM. As shown in the right panels, secreted IgM from BrM8 cells sedimanted...
primarily as completed polymers in a large peak at the lower end of the gradient. A small amount of secreted IgM at a size corresponding to that of monomers and half-monomers was also detected (Fig. 3, upper right), and these were well separated from the polymers. Analysis of the fractions containing secreted IgM polymers on denaturing gels (Fig. 3, lower right, fractions 10-19) revealed only completed, covalent polymers as there were no smaller IgM oligomers generated from these fractions. The presence of non-covalent polymers intracellularly but not in secretions, together with the demonstration that secreted assembly intermediates are well separated from the polymers on sucrose gradients, argues that secreted IgM assembly intermediates do not exit the cell as part of non-covalently assembled pentamer or hexamer complexes. Rather, they are secreted as monomers, dimers, and other individual assembly intermediates. Non-covalent polymers are also not secreted by cells expressing \( \mu \) chains in which Cys575 is mutated; these cells secrete IgM subunits as a consequence of this mutation (Wiersma and Shulman, 1995).

While the previous data showed that non-covalently assembled polymers are not secreted, they did not eliminate the possibility that some intermediates traverse the secretory pathway as part of non-covalent polymer complexes which dissociate into smaller oligomeric forms before secretion. We therefore asked whether non-covalently assembled IgM polymers are transported into a late compartment of the secretory pathway. BrM8 cells were metabolically labeled with \([\text{H}]\)galactose to tag those forms of IgM bearing N-linked glycans that have been processed in the \( \text{trans} \)-Golgi (Roth and Berger, 1982). Lysates from labeled cells were fractionated on a sucrose gradient (Fig. 4A). Most of the label was found in a peak of IgM corresponding in size to completed polymers, and a smaller amount was detected in a peak corresponding to monomers (Fig. 4A). As revealed on denaturing/non-reducing gels, the monomers (fractions 14-16) were well separated from the polymers (fractions 6-9) in the gradient and there was no evidence for galactose-labeled monomers in the polymer fractions (Fig. 4B,C). Thus, non-covalently assembled polymers are not transported into the \( \text{trans} \)-Golgi. Rather, a fraction of assembly intermediates, primarily monomers, mature through this organelle as individual components.

As a second approach to determine whether non-covalently assembled polymers mature through the secretory pathway, we asked if these complexes contain endo H resistant N-linked glycans. BrM8 cells were internally labeled with \([\text{S}]\)methionine and -cysteine and the cell lysates were then fractionated on a sucrose density gradient. A portion from the leading edge of the polymer peak (fractions 12-14) and fractions containing assembly intermediates, including monomers and half-monomers (fractions 26-34), were separately pooled and analyzed. \( \mu \)-containing proteins were immunoprecipitated, treated with endo H, and then resolved on 2-D gels. Both endo H resistant and endo H sensitive covalent polymers were resolved from the polymer-containing fractions (Fig. 5B). In addition, a trail of endo H sensitive intermediates, which represents the dissociation of immature non-covalent polymers in the denaturing agarose/SDS-PAGE first dimension (see Fig. 3), was also detected. However, there was no evidence for endo H resistant non-covalently associated polymers (Fig. 5B). Small amounts of endo H resistant assembly intermediates, primarily monomers, could be detected in fractions 26-34 (Fig. 5C). The fact that these mature intermediates were contained in the monomer peak and were well-separated from the polymers on sucrose gradients verifies that they are not derived from non-covalently assembled polymers. We conclude that non-covalently assembled polymers do not mature through the secretory pathway, and are therefore not a

![Fig. 4.](image-url)
vehicle for the transport of assembly intermediates. Thus, secreted intermediates arise from the ‘release’ and maturation of individual IgM assembly components.

**Endo H sensitive but not endo H resistant μs chains are sensitive to alkylation by NEM**

The fact that secreted IgM assembly intermediates transit the secretory pathway as individual components prompted us to assess the oxidation status of cysteines in immature and mature μs chains using the agent NEM, which efficiently and irreversibly alkylates free thiol groups (Chanat et al., 1993). BrM8 cells were solubilized in the presence of [3H]NEM, and a sample of culture supernatant was treated with [3H]NEM. In parallel, intracellular and secreted IgM was obtained from samples not alkylated with [3H]NEM. Following immunoprecipitation, μ-containing proteins were treated with or without endo H, resolved by reducing SDS-PAGE, and detected by autoradiography ([3H]NEM, Fig. 6A) or by western blotting with anti-μ (Fig. 6B). Reducing SDS-PAGE was used in order to directly compare the NEM sensitivity of intracellular endo H sensitive and endo H resistant μs. Only immature endo H sensitive μs was labeled with [3H]NEM (Fig. 6A). Mature, endo H resistant μs was present, as revealed by western blot analysis (Fig. 6B), but was not labeled with [3H]NEM (Fig. 6A). Similarly, the mature μs chains of secreted IgM were not labeled by [3H]NEM, but were revealed by western blotting (Fig. 6A,B). Therefore, neither secreted nor mature intracellular forms of IgM are sensitive to alkylation by NEM, a finding consistent with all forms of secreted and mature intracellular IgM having all cysteine residues, including Cys575, in an oxidized state.

**Fig. 5.** Maturing, intracellular IgM assembly intermediates are not derived from non-covalently assembled polymers. BrM8 cells were metabolically labeled with [35S]methionine and -cysteine and the cell lysates were then fractionated on a sucrose density gradient. (A) μ-containing proteins were immunoprecipitated from a sample of every second fraction and the amount of incorporated label determined by scintillation spectroscopy. The leading edge of the polymer-containing peak, fractions 12-14, and a region containing predominantly monomer and half-monomer subunits, fractions 26-34, (both marked with bold lines) were then pooled separately and immunoprecipitated for μ-containing proteins. The proteins were treated with endo H and then examined by 2-D gel analysis exactly as in Fig. 2. The portions of the gels containing the μs bands are shown in B and C. (B) Polymer-containing fractions 12-14; completed polymers are marked with a bracket. (C) Monomer/half-monomer-containing fractions 26-34; endo H resistant, mature assembly intermediates are marked by asterisks.

**Fig. 6.** Endo H sensitive, but not endo H resistant, μs chains contain exposed thiols. (A) BrM8 cells were lysed in the presence of [3H]NEM; a sample of culture supernatant was also treated with the labeled alkylating agent. Alkylated samples were pre-cleared with NRS. μ-containing proteins were then immunoprecipitated, treated with or without endo H, and resolved by reducing SDS-PAGE. Labeled proteins were revealed by autoradiography. The two intense bands detected in the NRS preclear of the supernatant sample are serum proteins which are labeled by NEM (data not shown). The faint bands which were detected in the anti-μ immunoprecipitate from the alkylated supernatant are not secreted μs chains as they were also detected by the NRS/Protein A-Sepharose preclear and migrated with faster mobility than did intracellular μs. (B) BrM8 cell lysates and culture supernatant were prepared in the absence of labeled NEM. μ-containing proteins were then prepared and resolved exactly as in A and revealed by western blotting using a labeled anti-μ probe. The gels shown in A and B were run in parallel in the same electrophoresis chamber.
DISCUSSION

IgM antibodies are generally thought to be secreted only as covalently complete pentameric and hexameric polymers under normal conditions (Davis and Shulman, 1989; Brewer et al., 1994a). However, our data demonstrate conclusively that incomplete polymers, especially IgM monomers, are also secreted at low levels. While the presence of incomplete polymers in secretions has been noted by others (Davis et al., 1989; Randall et al., 1990; Brewer et al., 1994b), our data indicate that the secretion of assembly intermediates is a normal and universal consequence of IgM biogenesis. Two lines of evidence indicate that secreted IgM intermediates arise from the transit of individual assembly intermediates through the Golgi. First, the profile of secreted assembly intermediates closely parallels the profile of individual assembly intermediates that mature intracellularly (Fig. 2). Second, non-covalently assembled polymers are not processed through the Golgi and are, consequently, not a vehicle for the transport of assembly intermediates through this organelle (Figs 3, 4, 5). Therefore, assembly intermediates, like completed polymers, are normally processed through the secretory pathway upon exiting the ER. Shachar et al. (1992, 1994) have also detected intracellular galactosylated monomers in antibody secreting cells that are, consequently, not a vehicle for the transport of assembly intermediates. Like completed polymers, these monomers are not processed through the Golgi. First, the profile of secreted assembly intermediates derived from the transit of individual assembly intermediates through the Golgi. Therefore, the fact that assembly intermediates are not processed through the Golgi underscores the importance of an effective system for retaining IgM assembly intermediates within the polymerization compartment, the ER (Tartakoff and Vassali, 1979; Brewer et al., 1994b; Bornemann et al., 1995).

A quality control mechanism in which proteins bearing free thiols are subject to retention in the ER has been suggested to govern the biogenesis of secretory IgM (Alberini et al., 1990; Sitia et al., 1990) as well as several other secretory proteins (Alberini et al., 1990; Kerem et al., 1993; Guenzi et al., 1994). While the molecular details of the thiol-mediated mechanism proposed to be responsible for their retention are not yet fully understood, a central element of the process is believed to involve the monitoring of the redox state of a single cysteine residue in each protein subject to its control, as has been described for Cys575 of the \( \mu \) chain (Sitia et al., 1990; Fra et al., 1993). Our data support the concept that the redox state of one or more cysteines is critical in determining whether secretory IgM, whether completely polymerized or not, is allowed to exit the ER to be secreted. First, there is no evidence that non-covalently assembled polymers mature through the secretory pathway (Figs 3, 4, 5), despite the fact that these complexes can be detected as part of the steady state distribution of intracellular polymerizing IgM (Fig. 3). Thus, the quality control mechanism(s) governing secretory IgM biogenesis demands more than the simple assembly of higher-order polymeric structures prior to release and transit through the secretory pathway. Second, in contrast to the vast majority of intracellular \( \mu \) chains which are contained within the ER, the \( \mu \) chains that have exited the ER to mature through the Golgi no longer contain free thiols sensitive to alkylation by NEM (Fig. 6). Together, these findings support the idea that it is the oxidation of all available cysteines, including Cys575, that releases secretory IgM from retention in the ER. While this is normally achieved by covalent polymerization of IgM into pentamers and hexamers, this also occurs in a fraction of assembly intermediates. As a result, these molecules meet quality control standards in the strict sense and, consequently, mature and are secreted as 'incomplete' polymers.

Based on the evidence that the ER is the primary oxidizing environment of the cell (Hwang et al., 1992), we predict that the oxidation of the important cysteine(s) occurs in the ER or, perhaps, in an ER-derived transport vesicle. There are several, not mutually exclusive, mechanisms that might account for the export of IgM assembly intermediates from the ER. First, a fraction of assembly intermediates may meet quality-control standards in the ER because all cysteines, including Cys575, become oxidized in the absence of complete polymerization. For example, the two partner \( \mu \) chains of an IgM monomer could form a disulfide bond between their corresponding Cys575 residues, an event which would be predicted to release the monomer from retention. Such a mechanism would be consistent with monomers and other whole-step intermediates being secreted more abundantly than half-step intermediates. While the quaternary structures of the secreted higher-order assembly intermediates are unknown, it is possible that at some frequency IgM can form dimers, trimers, and tetramers in which all C-terminal cysteines are oxidized, yielding structures characteristic of secreted polymeric IgA (Mestecky and Kilian, 1985). Alternatively, the Cys575 moieties on a fraction of assembly intermediates may be masked by free cysteine and/or glutathione present in the oxidizing milieu of the ER lumen, providing a means for the oxidation of all Cys575 residues of the small amount of secreted IgM and other half-step intermediates. Indeed, after submission of this paper, Reddy et al. (1996) published the results of mass spectroscopy studies of IgM with free cysteine and/or glutathione present in the oxidizing milieu of the ER lumen, providing a means for the oxidation of all Cys575 residues of the small amount of secreted IgM and other half-step intermediates. Indeed, after submission of this paper, Reddy et al. (1996) published the results of mass spectroscopy studies of monoclonal IgM and IgA, which all C-terminal cysteines are oxidized, yielding structures characteristic of secreted polymeric IgA (Mestecky and Kilian, 1985).
regulates secretory IgM biogenesis is an inherently imperfect process that allows small amounts of assembly intermediates to escape and mature through the secretory pathway. We propose that the imperfection lies not in the ability of the retention mechanism to recognize and retain free thiol-bearing IgM assembly intermediates within the ER, but rather reflects the absence of a mechanism to distinguish whether oxidized CySS575 moieties are contained within covalently completed polymers or within polymer intermediates. Importantly, this model suggests that assembly intermediates, at least those that become oxidized, are not stably associated with any resident ER chaperones that contain retention or retrieval sequences of their own (Pelham, 1995).

What are the consequences that arise from such an inefficiency in retention? While the low level release of assembly intermediates may reflect a tolerated inefficiency in IgM biogenesis that is of little or no consequence to the host, there is some correlative evidence suggesting that the presence of large amounts of secreted IgM assembly intermediates, such as those characteristic of low molecular weight IgM that accompanies their premature release from retention. The efficiency of cysteine-mediated intracellular retention determines the differential fate of secretory IgA and IgM in B and plasma cells. The efficiency of cysteine-mediated intracellular retention determines the differential fate of secretory IgA and IgM in B and plasma cells. Eur. J. Immunol. 24, 2477-2482.

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(Received 21 March 1996 – Accepted 19 June 1996)