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Immunology

Roles of heavy and light chains in IgM polymerization

[IgM secretion/μ chain/J chain/endoplasmic reticulum/chaperonin BiP (78-kDa glucose-regulated protein)]

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ABSTRACT **IgM antibodies are secreted as multisubunit polymers that consist of as many as three discrete polypeptides: μ heavy chains, light (L) chains, and joining (J) chains. We wished to determine whether L chains that are required to confer secretory competence on immunoglobulin molecules must be present for IgM to polymerize—that is, for intersubunit disulfide bonds to form between μ chains. Using a L-chain-loss variant of an IgM-secreting hybridoma, we demonstrated that μ chains were efficiently polymerized independent of L chains, in a manner similar to that observed for conventional μL complexes, and that the μ polymers incorporated J chain. These μ polymers were not secreted but remained associated with the endoplasmic reticulum-resident chaperone BiP (GRP78). This finding is consistent with the endoplasmic reticulum being the subcellular site of IgM polymerization. We conclude that μ chain alone has the potential to direct the polymerization of secreted IgM, a process necessary but not sufficient for IgM to attain secretory competence.**

IgM antibodies are secreted as polymeric molecules consisting of two or three discrete polypeptide chains (1, 2). Like other immunoglobulins, monomeric IgM has a basic subunit structure consisting of two heavy (H) chains (the μ chains) and two light (L) chains. However, in contrast to most other immunoglobulin molecules, IgM is efficiently secreted only if it is polymerized into pentamers or hexamers (3–5), which comprise five or six covalently associated μ_2L_2 monomeric subunits, respectively. Pentamers can also contain a third polypeptide, the joining (J) chain, that is disulfide-linked to two of the μ chains (6, 7).

The precise roles of each polypeptide chain in the polymerization process are not well defined. Incorporation of J chain into IgM is not required either for it to polymerize or for it to acquire secretory competence (3, 5, 8, 9). J chain does, however, regulate the complexity of polymeric IgM, in that high levels of J chain favor the formation of pentamers at the expense of hexamers (5, 10). L chains influence the transport of immunoglobulin molecules. In their absence, all H-chain isotypes remain in an early compartment of the secretory pathway, in part via their interaction with the H-chain-binding protein, BiP (GRP78), an endoplasmic reticulum (ER)-resident molecular chaperone of the Hsp70 heat shock protein family (11–14). To confer secretory competence on H chains of the μ isotype, polymerization of μL subunits into pentamers or hexamers must also occur before IgM antibodies can efficiently be secreted (3–5). Obviously, the μ chain is critical in this process, since it supplies the cysteine residues, including Cys^{575} in its secretory tailpiece, that are required for the covalent polymerization of IgM (1, 2). In addition, Cys^{575} also confers thiol-dependent retention of μ chains (4, 15), ensuring that only completely polymerized IgM antibodies, in which all Cys^{575} thiol groups are masked in disulfide bonds, are shuttled into the secretory pathway (4). Whether L chains contribute to this polymerization process is not known, however. Specifically, is the association of μ chains with L chains a prerequisite for μ chains to polymerize—that is, to form disulfide bonds between their Cys^{575} residues?

We established a system to examine the polymerization of secretory μ chains in the absence of L chains by taking advantage of a variant of an IgM-secreting hybridoma that has lost the production of L chain. We demonstrate that μ chains assemble with J chain into IgM-like polymers in the absence of L chains.

MATERIALS AND METHODS

Cell Lines and Subcloning. All cell lines were grown in complete RPMI medium (16). The hybridoma line NYCH.μκ was derived by fusing the B lymphoma NYC to the immunoglobulin-negative plasmacytoma Ag8.653 (17). To isolate μ-chain-loss variants, NYCH.μκ was subcloned by limiting dilution. Immunoglobulin production was determined by cytoplasmic immunofluorescence (16).

Antisera and Monoclonal Antibodies. Unlabeled and fluorochrome-labeled immunoglobulin-subclass-specific polyclonal goat and rabbit antibodies were purchased from Southern Biotechnology Associates. The polyclonal goat anti-mouse λ antiserum cross reacted with μ chains and was adsorbed twice on an IgM(κ) affinity column. Anti-λ specificity was tested by immunoprecipitating radiolabeled lysates from IgM(λ)- and IgM(κ)-secreting lines. The rat monoclonal antibody b7-6, a gift from Georges Köhler (Max-Planck Institut, Freiburg, Germany), reacts with the mouse μ-chain constant-region domain $C_\mu2$ (18) and was precipitated with a rabbit anti-rat IgG serum. The monoclonal rat anti-mouse κ antibody 187.1 (19) was a gift from Michael Julius (University of Toronto, Toronto) and the monoclonal rat anti-mouse BiP antibody (11) was provided by J. Kearney (University of Alabama, Birmingham, AL). These two antibodies were precipitated with *Staphylococcus aureus*. The anti-BiP serum used on Western blots was generated by immunizing a rabbit with a purified recombinant BiP fusion protein. The rabbit anti-mouse J chain antiserum was provided by Michael Parkhouse (Pribright Laboratory, Pribright, England).

Metabolic Labeling and Immunoprecipitation. Cells ($1–3 \times 10^6$) were starved for 1 hr in 1 ml of methionine-free medium

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Abbreviations: H, heavy; L, light; ER, endoplasmic reticulum; DTT, dithiothreitol.

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and then metabolically labeled with Tran^{35}S-label (1076 Ci/mmol; ICN; 1 Ci = 37 GBq) at 75 μCi/ml for 20–180 min. Cells were lysed on ice for 20 min in NET lysis buffer (20) with the sulfhydryl blocker iodoacetamide (100 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 μM), and leupeptin (40 μM). Proteins were precipitated from culture supernatants and lysates with antibodies against μ and L chains, followed by *S. aureus* prepared as described (21). Precipitates were washed (20), solubilized in the appropriate sample buffer, and analyzed by SDS/PAGE.

For pulse–chase experiments, cells (2×10^6 per ml) were starved in methionine-free medium for 30 min at 37°C and pulse-labeled for 15 min at 37°C in the presence of [^{35}S]methionine (100 μCi/ml, 1000 Ci/mmol) (Amersham) and 10 mM dithiothreitol (DTT). Cells (2×10^6) representing time zero of the chase were removed and washed in ice-cold phosphate-buffered saline (PBS) supplemented with the sulfhydryl blocker *N*-ethylmaleimide (20 mM) (22). The remaining cells were washed once in ice-cold medium with 2 mM methionine and then suspended at 2×10^6 cells per ml in warm complete medium with 2 mM methionine. Cells (2×10^6) were removed at indicated times, washed in PBS with 20 mM *N*-ethylmaleimide, and lysed in NET lysis buffer. Immunoglobulin proteins were analyzed as described above.

Gel Electrophoresis. One-dimensional SDS/PAGE (23), Western blot analysis (24), two-dimensional nonreducing–reducing SDS/PAGE (25), and nonreducing SDS/agarose/PAGE (26) were performed as described. The apparent molecular weights of protein markers vary among these gel systems. Radiolabeled proteins were detected by autoradiography or fluorography. The radioactive signals were quantified either by scintillation counting of dried gels in a blot analyzer (Betagen, Waltham, MA) or by laser densitometry of autoradiographs.

RESULTS

Characterization of a μ-Chain-Positive, L-Chain-Negative Murine Plasma Cell Line. NYCH.μκ hybridoma cells have all the characteristics of plasma cells, in that they secrete IgM (Fig. 1) and synthesize mRNA only for the secreted form of μ chains (data not shown). We isolated from NYCH.μκ a μ-only-producing line, NYCH.μ. NYCH.μ produces full-length μ chains but does not synthesize κ chains and does not secrete μ chains (Fig. 1). Southern blot analysis revealed that NYCH.μ does not produce κ chains because it has lost the active κ allele of NYCH.μκ (data not shown). By immunoprecipitating cellular lysates with an anti-λ antiserum, we also excluded the possibility that NYCH.μ had switched from κ- to λ-chain expression (Fig. 1).

We detected an additional protein with an apparent molecular weight slightly less than that of κ chains in anti-μ-chain but not in anti-L-chain precipitates from NYCH.μ lysates (Fig. 1). Western blot analysis confirmed the identity of this protein as J chain (see Fig. 4*B*), a 15-kDa protein that migrates aberrantly in SDS/polyacrylamide gels because of its acidic nature (27).

Polymerization of μ Chains in the Absence of L Chains. To determine whether μ chains in NYCH.μ cells formed disulfide-linked polymers in the absence of L chains, we resolved anti-μ precipitates by nonreducing SDS/agarose/PAGE. We detected in NYCH.μ samples a protein ladder of discrete bands, similar to that in NYCH.μκ cells (Fig. 2) and other IgM-secreting cells (26). Three major bands migrated at the bottom of the gel with apparent molecular masses of 72, 92, and 148 kDa. The 92-kDa and 148-kDa proteins corresponded to monomeric μ chains and μ_2 dimers, respectively. The mobilities of these bands were slightly faster than those of μκ and $\mu_2\kappa_2$ in NYCH.μκ cells (Fig. 2). These same two protein bands were also detected by Western blot analysis using anti-μ

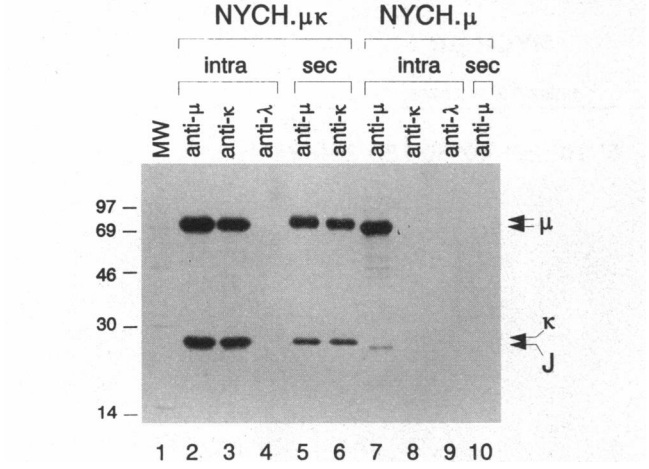

FIG. 1. Fluorograph obtained after SDS/PAGE of reduced ^{35}S-labeled immunoglobulin proteins produced by various NYCH clones. ^{35}S-labeled proteins were immunoprecipitated from equal aliquots of culture media ("sec") and cell lysates ("intra") with an anti-μ serum, an anti-λ serum, and monoclonal anti-κ antibodies; resolved in a reducing SDS/10% polyacrylamide gel; and detected by fluorography. The protein with an apparent molecular mass of 27 kDa that coprecipitated with μ in the NYCH.μ and NYCH.μκ samples (lanes 2, 3, and 7) was identified as J chain (see Fig. 4*B*). J chain could not be seen in secreted IgM (lanes 5 and 6), probably because the maturation of N-linked oligosaccharides caused it to migrate at a position where it was overshadowed by κ chains. The μ chains in secreted IgM (lanes 5 and 6) also migrated with a slower rate than intracellular μ chains, because of N-linked oligosaccharide maturation differences. Lane 1, ^{14}C-labeled protein molecular weight standards (molecular masses in kilodaltons are at left).

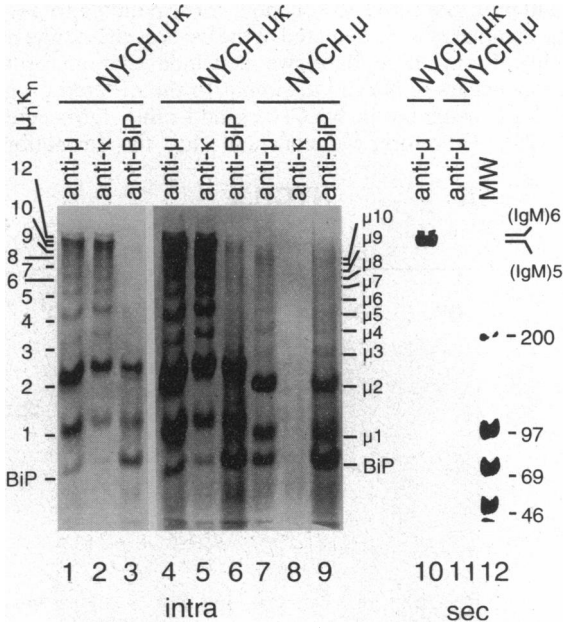

FIG. 2. Analysis of μ assembly in NYCH clones on a nonreducing SDS/agarose/polyacrylamide gel. [^{35}S]Methionine-labeled proteins were immunoprecipitated from aliquots of cellular extracts ("intra") and culture supernatants ("sec") with monoclonal anti-μ, anti-κ, and anti-BiP antibodies; separated in a nonreducing SDS/0.5% agarose/2.5% polyacrylamide slab gel; and detected by fluorography. Lanes 1–3 show a shorter film exposure of lanes 4–6. The mobilities of $\mu_n\kappa_n$ assembly intermediates (13) and ^{14}C-labeled protein molecular weight standards (lane 12) served as standards to determine apparent mobilities of the μ_n-containing assembly complexes in NYCH.μ. A half-logarithmic blot of apparent molecular weights of μ intermediates versus their migration distances revealed that each of the bands in NYCH.μ migrated with an apparent molecular weight that is consistent with a difference of one monomeric μ chain. Under these gel conditions, intracellular $\mu_{10}\kappa_{10}$ complexes in IgM-secreting plasma cell lines separate as a doublet (26).

antibodies (data not shown). The 72-kDa band appeared to be BiP, which is known to bind free μ (12). It had greater intensity in anti-μ precipitates from NYCH.μ than from NYCH.$\mu\kappa$, and it could be detected with an anti-BiP antiserum by Western blotting (data not shown).

In both NYCH.$\mu\kappa$ and NYCH.μ lysates, we detected additional bands above the $\mu_2\kappa_2$ and μ_2 complexes, each of which migrated with an apparent molecular mass that is consistent with a difference of a half-monomeric μL subunit in NYCH.$\mu\kappa$ and a monomeric μ chain in NYCH.μ. The most abundant bands corresponded to $(\mu_2\kappa_2)_5$ in NYCH.$\mu\kappa$ and to μ_{10}-containing complexes in NYCH.μ. Because we detected all possible μ assembly intermediates (μ to μ_{10}) in NYCH.μ, we conclude that μ polymerization is not compromised in the absence of L chain.

To compare the assembly kinetics of μ-containing polymers in the parental and the L-chain-loss variant cell line, we performed pulse–chase experiments. For each time point, intracellular μ-containing complexes were immunoprecipitated and analyzed by SDS/agarose/PAGE. To prevent covalent assembly of newly synthesized μ and κ chains during pulse labeling, we supplemented the medium with DTT, which rapidly and reversibly prevents disulfide bond formation in intact cells (28). As expected, only monomeric μ chains were detected after the DTT/[^{35}S]methionine pulse in both NYCH clones (Fig. 3).

The pattern of μ-containing intermediates during the chase period was very similar in both NYCH.μ and NYCH.$\mu\kappa$. The most abundant intermediates (μ_2 in NYCH.μ and $\mu_2\kappa_2$ in NYCH.$\mu\kappa$) reached maximal levels 10 and 15 min after initiation of the chase and disappeared with half-times of 120 and 140 min, respectively. Polymers corresponding to μ_{10} and $\mu_{10}\kappa_{10}$ complexes were detected at the earliest chase time point (5 min). From these data, we conclude that an ordered assembly occurs in NYCH.μ, similar to the ordered progression of μL assembly in NYCH.$\mu\kappa$ and other IgM-secreting cells (26). Therefore, we conclude that the formation of intersubunit disulfide bonds between μ chains does not depend on the presence of preformed μL complexes and that the absence of L chains does not alter the μ assembly process.

Incorporation of J Chain into μ Polymers. To determine whether J chain is disulfide-linked with μ complexes in NYCH.μ cells, we analyzed intracellular μ complexes by two-dimensional nonreducing–reducing SDS/PAGE. Free μ chains (μ) and larger μ complexes (μ_n) were aligned horizontally (Fig. 4*A*), indicating that μ chains in NYCH.μ form disulfide-linked homopolymeric complexes. In addition, a protein corresponding to the size of J chain was aligned vertically with only the larger μ complexes, indicating that this protein is covalently linked to higher-order μ complexes. The identity of this protein was confirmed by two-dimensional Western blot analysis using an anti-J chain antiserum (Fig. 4*B*). These findings further support the idea that μ chains polymerize normally in the absence of L chains.

Association of μ-Containing Complexes with BiP. In contrast to completely assembled IgM polymers in NYCH.$\mu\kappa$ cells, μ_{10}-containing complexes are not secreted by NYCH.μ cells (Figs. 1 and 2), probably because they are retained by BiP. When we immunoprecipitated proteins from a lysate of ^{35}S-labeled NYCH.μ cells with monoclonal anti-BiP antibodies and separated the proteins by SDS/agarose/PAGE, we recovered μ-containing complexes of all sizes that were identical to the complexes that can be precipitated with anti-μ antibodies (Fig. 2). In contrast, only a fraction of $\mu_n\kappa_n$ complexes in NYCH.$\mu\kappa$ was recovered in anti-BiP-precipitated material of an NYCH.$\mu\kappa$ cell lysate (Fig. 2). This result was expected, since most of the predominant BiP binding sites, namely the C_H1 domain (14), are already occupied by κ chains. However, we were surprised to detect $\mu_2\kappa_2$ complexes in anti-BiP-precipitated material at all, because all classical BiP binding sites—that is, the C_H1 domains of μ chains—in these $\mu\kappa$-containing complexes should be saturated by L chains. It is possible that a component of what appears to be $\mu_2\kappa_2$ in reality contains $\mu_2\kappa$ complexes that would still be associated with BiP.

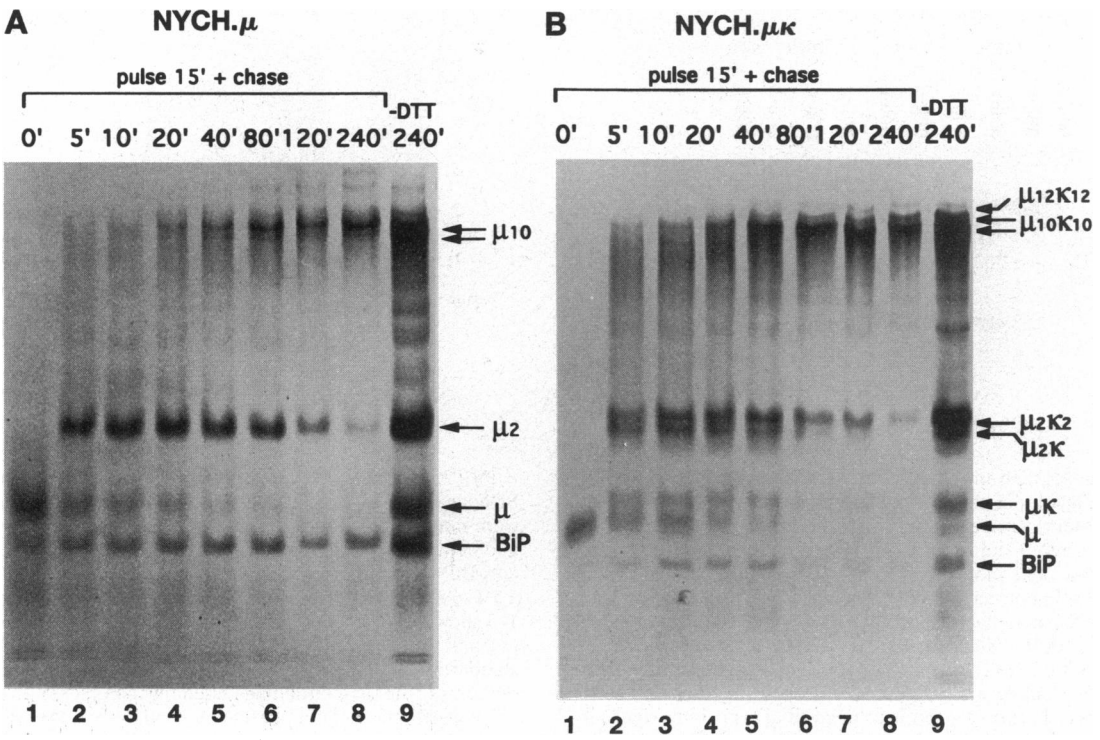

FIG. 3. Kinetic analyses of intracellular μ assembly in NYCH clones. NYCH.μ (*A*) and NYCH.$\mu\kappa$ (*B*) cells were labeled for 15 min with [^{35}S]methionine in the presence of DTT and then incubated with nonradioactive methionine for the indicated chase periods. Intracellular μ-containing complexes were precipitated and separated as described in Fig. 2. Intracellular μ complexes isolated from cells continuously labeled in the absence of DTT were loaded in lane 9.

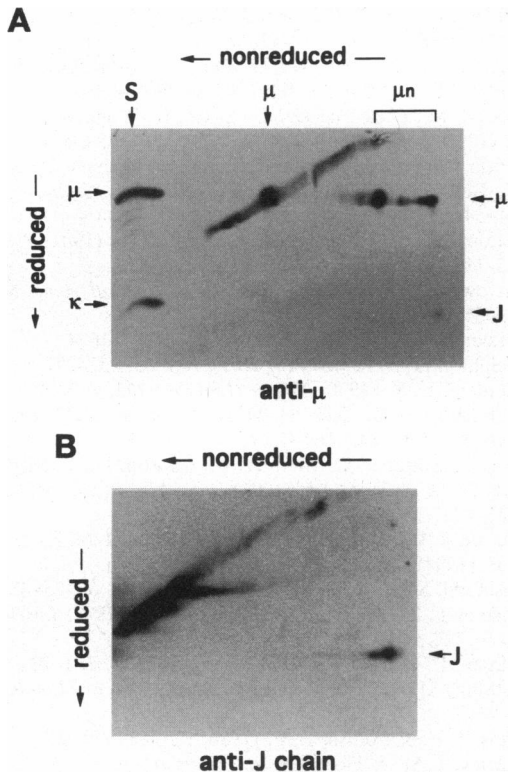

FIG. 4. Analyses of intracellular μ- and J-chain assembly in NYCH.μ cells. (*A*) Anti-μ-precipitated ^{35}S-labeled proteins were first separated in a nonreducing SDS/7.5% polyacrylamide gel and then in a reducing SDS/12.5% polyacrylamide slab gel and detected by fluorography. Reduced ^{35}S-labeled IgM from NYCH.μκ was applied in lane S and served as a molecular weight marker to determine the position of J chain. (*B*) Anti-μ-precipitated proteins were first separated as described in *A* and then transferred to a nitrocellulose membrane. J chain was detected with rabbit anti-J chain and ^{125}I-labeled goat anti-rabbit IgG antibodies. J chain was not detected when the filters were developed only with secondary antibodies (data not shown).

This would explain the relatively lower abundance of these complexes in anti-BiP relative to anti-μ precipitates. In summary, these data indicate that BiP remains associated with μ chains, even after their polymerization. These results indicate that polymerization of μ chains occurs in a BiP-containing compartment, presumably the ER.

DISCUSSION

Acquisition of secretory competence by proteins is a complex process in which various enzymes and molecular chaperones participate to process N-linked glycans, catalyze disulfide-bond formation, and facilitate the proper folding of newly synthesized polypeptide chains (29, 30). For multimeric proteins an additional level exists: only properly oligomerized component polypeptide chains transit the secretory pathway (31, 32). In the case of antibody molecules, the L chain confers secretory competence on H chains; this requirement is absolute, as wild-type H chains are never secreted in the absence of L chains (11, 12, 14, 33–35). In the case of IgM antibodies, efficient secretion requires the oligomerization of primary subunits into higher-order polymers (1, 4, 26), which depends on disulfide bonds involving cysteine residues of μ chains. However, it is not known whether the potential of μ chains to polymerize is dependent on their ability to be secreted—that is, to associate with L chains. Therefore, we sought to determine whether the oligomerization of μ chains requires the presence of L chains or whether this process proceeds independent of secretory competence.

We have shown here that the association with L chains is not required for μ chains to polymerize, since they form higher-order polymers in the absence of L chains. By several criteria, our data support the idea that the polymerization of μ chains in NYCH.μ cells reflects an ordered assembly and is not an abberant aggregation of mis- or unfolded μ chains. First, the analysis of the steady-state distribution of μ-containing complexes in NYCH.μ cells revealed an assembly ladder that was similar in composition to that detected in parental IgM-secreting NYCH.μκ cells, as well as in other IgM-secreting cells (26). Second, pulse–chase experiments revealed that, in the absence of L chains, μ-chain assembly occurred in an ordered process similar to that observed for the formation of IgM polymers. Finally, the μ-containing polymers in NYCH.μ incorporated J chain. These findings are consistent with an ordered assembly of μ chains in the absence of L chains.

As expected, both monomeric μ chains and higher-order μ-containing polymers in NYCH.μ cells were associated with BiP, indicating the ER as the site of IgM polymerization. However, our finding does not exclude a role of other intracellular compartments in IgM polymerization. BiP can migrate from the ER into the cis-Golgi compartment, where it is retrieved by the Lys-Asp-Glu-Leu (KDEL) receptor via its carboxyl-terminal KDEL sequence and recycled back into the ER (36). Whether this recycling is of physiological relevance with respect to the fate of BiP ligands remains to be established. Our findings are in agreement with studies that demonstrated that IgM polymers are completely assembled prior to the mid-Golgi compartment (4, 26).

BiP binds to unfolded parts of a polypeptide chain (reviewed in ref. 13) and, in the absence of L chains, it remains associated with H chains (12). However, this does not imply that the entire H chain is unfolded. While we have not directly assessed the folding status of μ chains in NYCH.μ cells, it seems unlikely that competence to form defined assembly intermediates in a stepwise manner and to incorporate J chain could be a property of misfolded μ chains. Instead, while domains near the amino terminus, including the C_H1 domain as the major BiP binding site (14), may not achieve their proper folding, domains in the carboxyl-terminal part, including the region that contains the important Cys^{575} residue, may be properly folded. In support of this, γ chains can bind protein A (12), which usually requires contact with both the folded C_H2 domain and the folded C_H3 domain (37), indicating that BiP-bound H chains might already exhibit in their carboxyl-terminal portion the three-dimensional structure of a mature antibody. Further, it has been shown that one domain of a BiP-associated L chain has already formed one intradomain disulfide bond, whereas the other domain is not yet oxidized (38).

Our finding that μ chains assemble into IgM-like polymers in the absence of L chains further clarifies our understanding of the respective role of each individual polypeptide chain of IgM polymers in their synthesis and secretion. Single μ chains have the potential to covalently polymerize into higher-order forms, a process that masks Cys^{575} thiol groups that are required for thiol-mediated retention of μ chains (4, 15). Nevertheless, the μ-containing complexes are retained by BiP until all C_H1 domains have paired with the constant domain of a L chain. Thus, while L chains are not required for μ chains to polymerize, they are critical in ensuring that polymeric IgM is transported through the secretory pathway. The third component, the J chain, does regulate the final composition of polymeric IgM, as high levels of J chain favor the synthesis of pentamers at the expense of hexamers (5, 10). Consequently, J chain exerts an influence on the biological potency of secreted IgM, since pentamers are 20-fold less efficient than hexamers in mediating the activation of the complement

cascade (3, 9). Together, the three chains cooperate through covalent association in the generation of discrete, functional IgM antibodies that can be secreted, a process that almost certainly involves an assembly machinery including chaperones and enzymes such as protein disulfide-isomerase (39).

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