ASSEMBLY AND SECRETION OF ANTIBODIES DURING B CELL DEVELOPMENT*

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All antibody classes (IgM, IgG, IgA etc.) share the basic H_2L_2 structure, and are produced in two forms during B cell development: as integral membrane proteins on the surface of B lymphocytes, where they act as antigen receptors, or as soluble effector molecules secreted by plasma cells. The topological problems related to this dual function of antibodies were explained in the early 80s by the finding that membrane and secreted Igs contain different heavy (H) chains, produced by alternate RNA processing. Heavy chains destined for membrane insertion (Hm) have a stretch of hydrophobic amino acids in their C-terminal end, which allow their insertion in the lipid bilayer. In contrast, hydrophilic peptides are found at the carboxyterminal ends of secreted H chains (Hs) (Alt et al., 1980; Early et al., 1980; Rogers et al., 1980; Kikutani et al., 1981; Sitia et al., 1982, 1985).

A problem that B cells face is that of synthesizing surface receptors capable of tranducing the desired signal(s) upon encounter with antigen. The absence of tyrosine-kinase or other signalling motifs in the cytoplasmic portions of Hm chains implies the association of the Hm_2L_2 complex with other molecules (Weiss and Littman, 1994). In contrast, plasma cells can be seen as short lived antibody factories. Each of them is able to produce thousands of molecules per second, a formidable achievement if we consider that antibodies, like all heteropolymeric molecules, are continuously checked by stringent "quality control" systems (see below). Far from being simple processes, the assembly and intracellular transport of functional antibodies in sufficient quantities and to the desired location are remarkable tasks for cells of the B lineage. Understanding the basic features of the antibody factory is of general relevance and may allow important biotechnological applications.

Quality control of newly synthesized proteins

The problem of protein folding is often referred to as the "second genetic code". How does a linear sequence of amino acids generate a functional three-dimenssional structure? The issue is further complicated for oligomeric proteins, which require the correct assembly of several subunits. Although the three dimensional structure of proteins is dictated by their amino acid sequences (Anfinsen, 1973), it has become increasingly clear that folding, assembly and oligomerization are catalyzed in vivo by a family of conserved "chaperone" molecules (Gething and Sambrook, 1992). These process are tightly linked to "quality control" events, which ensure that only proteins that attain the proper tertiary and quarternary structure reach their final destination. This is particularly evident in the case of secretory or membrane proteins, like the Igs, which reach the extracellular space after a long journey through the organelles of the exocytic compartment (Gaut and Hendershot, 1993; Hammond and Helenius, 1994).

The H and L chains have a N-terminal leader sequence (Milstein et al., 1972; Von Heijne, 1990) and are co-translationally translocated into the endoplasmic reticulum (ER). It is in this organelle that membrane and secretory proteins begin to fold, assemble and oligomerize, while being subject to extensive quality control (Hurtley and Helenius, 1989; Pelham, 1989; Klausner ans 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; Gaut and Hendershot, 1993).

Ig assembly in B and plasma cells

Our understanding of the folding, assembly and polymerization pathways of newly synthesized Igs in cells of the B cell lineage can be summarized as follows (see Wall and Kuehl, 1983; Day, 1990; Carayannopoulos and Capra, 1993 for extensive reviews):

1) H and L chains are synthesized on separates classes of polysomes.
2) H and L chains have a N-terminal leader sequence and are synthesized on ribosomes bound to the endoplasmic reticulum (ER) membrane.
3) H and L are co-translationally translocated into the lumen of the ER.
4) Subunit folding begins co-translationally and occurs independently for H and L.
5) Folding proceeds vectorially from the N-terminal towards the C-terminal domains.

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6) Folding proceeds assembly.
7) H and L assembly and H-H dimerization are also very rapid events, their order depending on the isotype and the size of the L chain pool.
8) Plasma cells generally produce an excess of L chains.
9) Free L chains can be secreted, while free H are retained and degraded intracellularly.
10) Polymerization is slow, and restricted to plasmacytoid cells.

Folding starts co-translationally and proceeds from the V to the C domains. This vectoriality might be important to prevent aberrant intra-chain disulphide bond formation and domain folding (Bergman and Kuehl, 1979 a, b). In addition, the rapid folding of individual domains might also prevent the backward movement of the growing chain from the ER lumen into the cytosol (Ooi and Weiss, 1992). Glycosylation and interactions with other proteins in the ER are also likely to play a role in tethering the newly made protein within the ER (Nicchitta and Blobel, 1993). However, folding need not occur co-translationally. Several lines of evidence demonstrate that both intra- and inter-chain disulphide bonds can be formed in Igs also after translation is completed (Valetti and Sitia, 1994, and references therein).

An intriguing observation is that in B lymphocytes producing both membrane and secretory chains, obviously of the same class, hybrid Hm-Hs moleculess are rarely detected (Oi et al., 1980; Word and Kuehl, 1981; Sitia et al., unpublished data). A possible explanation to this phenomenon may be that the rate of Ig synthesis in B cells is much lower than in myelomas. At low concentration, dimerization may be restricted to H chains synthesized on the same polysome. Structural or topological considerations are less likely, since H-H dimerization generally precedes chain termination, thereby occurring when H chains are still bound to the ER membrane, and perhaps when the different C-terminal peptides have not been synthesized. Alternatively, Hm-Hs hybrids may be rapidly edited by the quality control mechanisms in the ER.

Retention of free H chains

It has been long known that in the absence of L chanins, H chains are not secreted. For instance, myeloma variants which lose H chains but still produce and secrete L chains are easily isolated, while loss of L is a rare event, that yields unstable clones which retain and degrade the H chain intracellularly (Kohler, 1980). An analogous situation is encountered in the clinics when the monoclonal proteins produced by myeloma patients are analyzed. While the presencce of free L chains in the blood and urines of patients (the Bence-Jones protein) is a most frequent and diagnostic finding, very few cases have been described where the malignant clone produce and secrete free H chains. In these "Heavy Chain Disease" patients, the monoclonal H chain is always characterized by extensive deletions, which invariably encompass the C_H1 domain (Seligman et al., 1979). A unifying model explaining these observations became available when it was found that in the absence of L chains, newly synthesized H chains are bound to BiP, an abundant ER chaperone molecule. BiP, also called grp78, was first noted non-covalently bound to H chains in pre-B cells, which do not make L (Haas ad Wabl, 1983). It was therefore called the "H chain Binding Protein" (BiP), and thought to be involved in allelic exclusion. Later, it became clear that BiP is present in the ER of all cells, including yeast, and binds to misfolded or un/misassembled proteins, thereby preventing their aggregation and precipitation (Bole et al., 1986; Häas, 1991; Flynn et al., 1991; Melnick et al., 1994; Frydman et al., 1994). Hendershot and coworkers showed that i) free H chains can be secreted on deletion of the C_H1 domain (1987) and ii) L chains can displace BiP and assemble with H (Hendershot, 1990). While the first observation explains the situation encountered in Heavy Chain Disease, the second experiment indicates that BiP plays an important role in the normal Ig assembly pathways, preventing the aggregation of free H chains, as well as "presenting" them for interactions with L. As an efficient "chaperone", BiP interacts with the young, inexperienced protein, keeping it away from bad companies (precipitation, degradation) until the times are ripe for marriage (H-L assembly).

Secretion of free L chains

While H chains entirely obey to the rules of quality control, L chains are somewhat an exception as they can be secreted also in the unassembled state. The hydrophobic surfaces of V_L and C_L domains, interacting with V_H and C_H1 in assembled Igs, can also be masked by homodimerization. It is well known that Bence-Jones proteins are often present as L_2 dimers, either covalent or non-covalent (Milstein, 1965). Provided that the tendency to homodimerization is weaker than that to form H-L pairs, we may see how Ig assembly is catalysed reducing the risk of excessive intracellular accumulation (see Russell Bodies below). In the antibody factory, the goal is to optimise H-L assembly preventing the secretion of free H chains, which could alter monoclonality, complete with Ig for effector functions, and precipitate intra- and extra-cellularly. The solution is to create a vast pool of L chains, ready to accomodate all newly synthesized H. To do so, plasma cells synthesized L chains in excess, and retain them intracellularly until a sufficient concentration is obtained. The excess are secreted as L_2 or even as free L (see below), since these molecules carry no effector function and can be easily cleared by the kidney.

There are L chains which are not secreted unless assembled to H, the prototype being the NS-1 K chain (Kohler et al., 1976). In the absence of H, these chains are rather stably bound to BiP and eventually degraded intracellularly (Dul and Argon, 1990; Knittler and Haas, 1992; Gardner et al., 1993). However, even normal L chains transiently interact with BiP. What differs in the two classes of L chains is the half lives of the L-BiP complexes: a few minutes in wild-type, secreted L, hours in the case of the non-secreted mutants. L chains bind to other ER chaperone, GRP94, the homolog of cytosolic hsp90 and Erp72, an oxidoreductase that is abundantly expressed in plasma cells (Wiest et al., 1990). While BiP preferentially associates with early disulphide intermediates of L, GRP94 and Erp72 seem to bind to more mature L chains, in which the two intradomain disulphides are formed. The sequential interaction with different chaperones might be a general feature in the maturation of oligomeric proteins in the ER.

Developmental control of IgM secretion

The fate of secretory IgM varies dramatically during B cell development. Indeed, while plasma cells secrete this isotype very effiiciently, B cells retain and degrade intracellularly virtually all

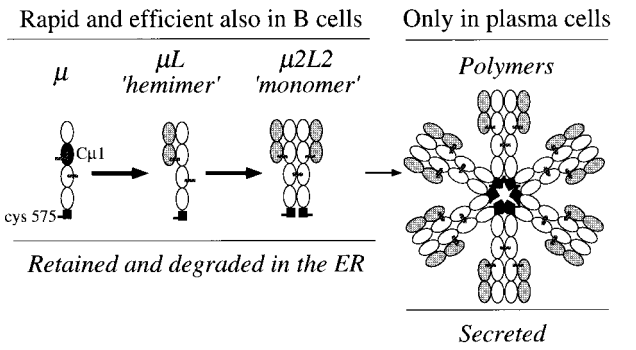

Fig. 1. Hierarchy of the events coupling the structural maturation of newly synthesized IgM to their intracellular transport. At least two retention elements are involved in the quality control of this isotype: first, the Cμ1 domain controls H-L assembly, second Cys575 is in charge of verifying that only polymers proceed to the Golgi. The logic of quality control is similar in both cases. It is assembly or polymerization which mask the retention element, allowing secretion. Pentamers with J chain or hexamers devoid J chain can be secreted by both lymphoid and non-lymphoid cells (Cattaneo and Neuberger, 1987; Randall et al., 1992 a, b). Mutant IgM lacking Cys575 are secreted also by pre-B as well as by B cell lymphomas, indicating that thiol-mediated retention is operative at all stages of B cell development in controlling IgM transport (Bornemann et al., 1997)

Tab. 1. Sequences of the Carboxyterminal Tailpieces of Secretory Immunoglobulins.

a) Conservation of the μs tailpiece during evolution[a]

Species	μ tailpiece * ** * *
Human	GKPTLYNVSLVMSDTAGTCY
Dog	----------L---------
Mouse	----------I----G----
Rabbit	----------S---
Chicken	--ASAV-----LA-S-AA--
Xenopus	----NV-----L-- -
Shark	---SFV-I--ALL--VNS-Q

b) Different heavy-chain isotypes in the mouse[b]

Isotypes	Tailpiece sequence	Serum form
μ	GKPTLYNVSLIMSDTGGTCY	only polymers
α	----NVS--Y---EGD-I--	oligo+monomers
τ	--	monomers
ε	--N-SLRP	monomers
δ	-CYH-LPE-DGP-RRPDGPALA	monomers

c) Insertion of a charged residue upstream Cys 575 induces secretion of unpolymerized IgM

Tailpiece sequence secreted[c]	% intermediates
GKPTLYNVSLIMSDTGGTCY	< 5
----------D----	30
----------D---	49
----------D--	68
----------E----	21
----------K----	27
----------S-	>95

[a,b] **See Sitia et al. (1990) and references therein. Dashes indicate identical amino acids. Asterisks denote conserved residues. N-linked glycosylation sites are underlined.**
[c] **IgM secreted as intermediate oligomers, monomers or hemimers (%). See Guenzi et al. (1994).**

μs_2L_2 complexes they synthesize (Sidman, 1981; Sitia et al., 1988). This is not due to a defective secretion in general, as surface Ig positive B cells secrete monomeric immunoglobulins, such as IgG or IgE (Sitia, 1985; Sitia et al., 1985, 1988) or mutant IgM in which the transmembrane portion of μm chains has been deleted (Sitia et al., 1990). Rather, IgM secretion is controlled at the level of assembly. The structure of secretory IgM is schematized in Figure 1. Two oligomeric forms, either hexamers or pentamers containing a J chain, are secreted by plasma cells (Davis et al., 1989 a; Sitia et al., 1990; Randall et al., 1992 a; Brewer et al., 1994). Thus, unlike IgG or other "monomeric" Igs, μ_2L_2 must further assemble to be transported through the Golgi. This is due to the presence of the so-called "secretory tailpiece" at the C-terminus of μ chains. Of the five classes of antibodies, only IgM and IgA — the two isotypes present in the blood and in secretion as polymers or oligomers — contain this 20 amino acid extension (Table 1). Within the μs tailpiece, a conserved cysteine in the penultimate position forms the covalent bonds linking the H_2L_2 subunits into a polymer with or without J chains. Not only is this residue (Cys575) essential for IgM polymerization, but it is also responsible for the selective retention of unpolymerized μ_2L_2 subunits. Indeed, mutant IgM whose μs chains lack Cys575 can be secreted as μ_2L_2 monomers, demonstrating that polymerization per se is not necessary for secretion. Thus, in the case of IgM secretion, selectively seems to be achieved not by the expression some "positive" transport signal on structurally mature proteins, but by the masking of retention elements on assembly intermediates. Accordingly, when the μs tailpiece containing Cys575 is added to the C terminus of $\tau_2\beta$ chains or to the lysosomal protease cathepsin D, he resulting chimeric molecules (IgG2bμtpCys or CDMμtpCys) are retained intracellularly by myeloma and Cos transfectants, respectively. Again, replacing the critical cysteine residue with serine restores secretion (Sitia et al., 1990; Fra et al., 1993; Isoioro et al., 196). The few CDMμtpCys molecules found in the supernatants are covalent dimers, confirming that Cys575 in the μs tailpiece can be used as a portable retention/dimerization motif. In addition, they reveal a third function for this element, that of inducing degradation of molecules which fail to assemble. When cathepsin D is retained in the ER by appending a KDEL motif at its C-terminus (Munro and Pelham, 1987), it is stable in the ER. This demonstrates that retention per se does not lead to degradation. Thus, specific signals must mark the molecules destinated for degradation.

For many years it has been assumed that the degradation of unfolded preteins occurs within the ER itself, or within a specialized subregion of this compartment. Thus, the involvement of ubiquitin and proteasomes in the disposal of membrane as well as secretory proteins (e.g. CFTR in human cells and CPY in yeast) (Ward and Kopito, 1996; Hiller et al., 1996) came a surprise. As proteasomes are localized in the cytosol, these observations introduce another unexpected requirement for the process of degradation to occur, that is the translocation of ER membrane (retrotranslocation or dislocation). Recent results in our laboratory, in agreement with studies on other system (Ward et al., 1995; Bierder et al., 1996; Hiller et al., 1996; Qu et al., 1996; Wiertz et al., 1996 a,

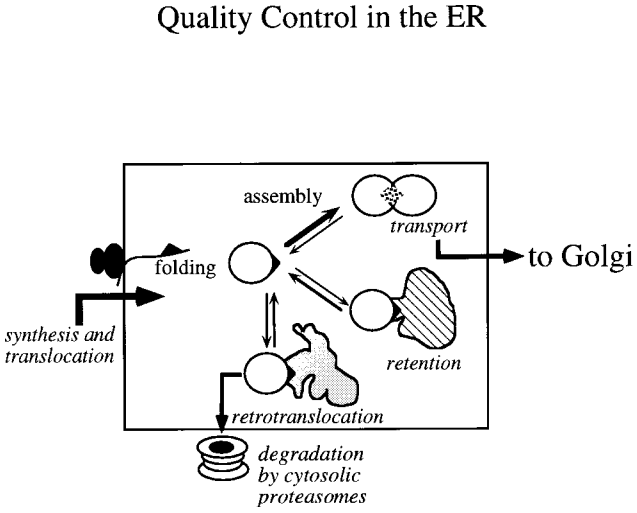

Fig. 2. Colocalised determinants for assembly, retention and degradation explain quality control Co-translational translocation into the ER, folding, assembly and transport to the Golgi are sequential events in the life of newly synthesized proteins. Unassembled subunits interact transiently with BiP, PDI and/or other resident proteins in the ER (striped) via the same element(s) that also mediate assembly and degradation (the dark triangle). Oligomerization will mask the signal (now in light grey) and allow transport. The degradative pathway is mediated by still unidentified molecules (drawn in grey in the scheme), which act as sorting devices activating a process of "retrotranslocation" and eventually delivering the polypeptide to cytosolic proteasomes. Modified from Fra and Sitia (1993).

b) demonstrate that also μ chain are degraded by cytosolic proteasomes. While inhibitors of lysosomal proteasse are not effective, lactyycystin and ALLN, two proteasome inhibitors, prevent the degradation of IgM by cells of the B lineage efficiently and rapidly assemble μ_2L_2 complexes, these must be dissociated to allow the retrotranslocation of μ and L chains. An interesting observation is that L chains that were part of a μ_2L_2 complex whose μ chains are rapidly degraded productively recycle to be reassembled with newly synthetized μ chains during the chase (CM and RS, unpublished results). This suggests that the targeting to degradation is extremely selective and disassembly does not occur in a dead-end ER subregion.

Taken together the above findings demonstrate that Cys575 may act as a three way switch, mediating assembly, retention and degradation of both IgM and cathepsin D chimeras (Figure 2). The existence of colocalized "signals" might be a general feature of the quality control of newly synthesized proteins (Bonifacino and Lippincott-Schwartz, 1991; Fra and Sitia, 1993, and references therein).

Biogenesis of Russell bodies

The crucial role of degradation in maintaining homeostasis within the secretory compartment is highlighted by the case of Russell bodies (Russell, 1890). These are dilated cisternae of the ER typical of Mott cells, frequently found in myelomas (Mott, 1905). Valetti et al. (1991) demonstrated that they are the result of mutations in the Ig that affect their intracellular transport. However, not all non-secreted mutants induce the formation of Russell bodies, but only those which are not degraded from the ER. Russell bodies are not tissue specific, as they are also induced in the rat C6 glioma cell line transfected with μ C_H1 and L genes. It thus appears that this phenotype represents a general response to the accumulation of abundant, nondegradable proteins that fail to exit from the ER.

Thiol-mediated retention

The role of disulphide interchange reactions in controlling protein transport is emphasized by the observation that membrane permanent monovalent reducing agents (i.e. 2-mercaptoethanol (2ME) and N-acetyl cysteine) induce secretion of unpolymerized IgM by pre-B, B and plasma cells (Albertini et al., 1990). Also the retention of IgG2bμtpCys and CDMμtpCys is overcome by 2ME (Fra et al., 1993; Isidoro et al., 1996). A likely explanation is that the reducing agent competes for the recognition of the free thiol group involved in retention. Other mechanisms of retention exist, such as the recognition of hydrophobic patches by BiP and/or other ER resident chaperones. Indeed, 2ME does not induce the secretion of proteins retained intracellularly by means other than disulphide interchange (i.e. BiP, free H chains, CDM-KDEL).

The amino acid context in which a given cysteine residues determines its efficacy as an ER retention element

Thiol-mediated retention mechanisms similar to those regulating IgM secretion have been described for acetylcholinesterase, an enzyme secreted only as disulphide-linked homodimers (Kerem et al., 1993) and Ron, a tyrosine kinase membrane receptor (Collesi et al., 1996). Yet, there are proteins which are secreted with unpaired cysteine(s). How do these proteins escape retention? Experiments on IgA revealed that the amino acid context surrounding the critical cysteine modulates its efficiency as a retention element. In particular, it is the presence of an acide residue upstream the α chain C-terminal cysteine that allows secretion of monomeric IgA by plasma cells.

A similar situation may explain the secretion of monomeric Bence-Jones proteins. As showed above, L chains are secreted also in the absence of H, either as covalent homodimers or as monomers (Knittler and Haas, 1992; Gardner et al., 1993). In the case of α chains, covalent dimers utilize Cys214, which also mediates the H-L disulphide in Ig molecules. In secreted monomers, however, Cys214 does not react with N-etylmaleimide, suggesting that it is covalently bound to a small moiety, perhaps a free cysteine (Milstein, 1965). The inability of Cys214 to act as an ER retention element might reflect the proximity of an acidic residue (Glu213), a constant feature of murine and human L chains. Indeed, replacing Glu213 with Gly or Lys inhibits secretion of α chains (Reddy et al., 1996). Thus, in both H and L chains, the presence of a charged residue upstream the relevant Cys is sufficient to reduce the efficacy of the retention mechanism(s). These results show a simple mechanism — the recognition of exposed thiols on assembly intermediates — can be precisely regulated, so as to achieve a fine tuning of retention/degradation and secretion (Guenzi et al., 1994).

Sequence diversification around the critical cysteine residue(s) is not the only mechanism by which thiol mediated retention can be modulated. In fact, monomeric IgA can be secreted by myeloma, but not by B lymphoma cells (Sitia et al., 1982, 1985), implying the existence of cellular factors regulating retention during B cell differentiation. Again, Xeopus oocytes behave like B cells, retaining and degrading intracellular IgA monomers (Carelli et al., 1997).

Thiol-based retention becomes of little if any importance in or beyond the Golgi

By exploiting two different reducing agents, 2ME and DTT, with different effects on Ig transport and assembly, it has been possible to demonstrate that also the thiol-based quality control mechanisms, so effective in the ER, become of little importance in or beyond the Golgi. Once proteins have reached the Golgi, they can proceed through the distal sections of the secretory apparatus also in the unassembled state, indicating that thiol-mediated is not operative beyond the ER (Valetti and Sitia, 1994).

That IgM polymerization normally occurs in the ER is demonstrated by analyses of the μ chain oligosaccharides (Cals et al., 1996). Furthermore, polymers containing BiP linked to endo-H sensitive μ chain have been demonstrated in cells lacking L chain (Bornemann et al., 1995).

Why don't B cells secrete IgM?

In the absence of antigen, Ig secretion would be deleterious for resting B cells, as it would drastically reduce their chances of binding antigen at low concentration via their membrane receptors. The particular structure of the μ chain gene — the only in which the exon encoding the secretory tail is not contiguous to the last constant domain — makes synthesis of μs molecules a rare event. Why don't B cells exploit the possibility of splicing out all μs exons to prevent IgM secretion? Either splicing is necessarily leaky (upon antigen binding B cells must rapidly differentiate into antibody factories releasing thousands of Igs per second) or the intracellular pool of $\mu s_2 L_2$ may represent a ready to use weapon when the presence of antigen is sensed. In contrast, in secondary responses, constitutive Ig secretion might favour selection of those clones in which the process of somatic mutation has increased the either the K_{eq} or the K_{on} of the antibody (Foote and Milstein, 1991).

At present, the reasons underlying the failure of B cells to secrete IgM or IgA are unclear. Recent results from our laboratory (Carelli et al., 1997 a) demonstrated that: i) like B cells, Xenopus laevis oocytes fail to polymerize and secrete IgM. ii) IgM polymerization can be induced in B cells by transient exposure to reducing agents. This B cells do not lack any structural component for polymerization.

Perhaps the most attractive possibility is that the redox potential within the ER is tightly regulated during B cell development, explaining the differential tendency to polymerization. Rather little is known at present on how redox gradients between subcellular organelles are maintained in living cells. Particularly exciting possibilities is the recent finding that the formation of disulphide bonds in the ER alter the redox in this compartment, and induce the production and the secretion of small thiols, mainly cysteine and glutathione (Carelli et al., 1997 b). These findings suggest that the level of Ig synthesis are linked to the maintainance of the redox within the secretory compartment. It will be of great interest to determine whether cellular factors involved in redox regulation are differentially expressed during B cell differentiation.*

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