Russell Bodies: A General Response of Secretory Cells to Synthesis of a Mutant Immunoglobulin Which Can Neither Exit from, Nor Be Degraded in, the Endoplasmic Reticulum

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Abstract. Dilated cisternae of the ER resembling Russell Bodies (RBs) are induced in light (L) chain producing myeloma cell lines by transfection of a μ heavy (H) chain gene lacking the first constant domain ($\mu\Delta$CH1). RBs do not appear to be tissue specific, since they are also induced in a rat glioma cell line transfected with $\mu\Delta$CH1 and L chain genes.

Efficient RB biogenesis requires H-L assembly and polymerization. The mutant Ig is partially degraded in a pre-Golgi compartment. The remnant, however, becomes an insoluble lattice when intersubunit disulphide bonds are formed. The resulting insoluble aggregate accumulates in RBs. Replacing the COOH-terminal cysteine of $\mu\Delta$CH1 chains with alanine reverses the RB-phenotype: the double mutant $\mu_{ala}\Delta$CH1 chains assemble noncovalently with L and are secreted as H2L2 complexes. Similarly, secretion of $\mu\Delta$CH1 chains can be induced by culturing transfectant cells in the presence of reducing agents. The presence of RBs does not alter transport of other secretory or membrane molecules, nor does it affect cell division. Resident proteins of the ER and other secretory proteins are not concentrated in RBs, implying sorting at the ER level. Sorting could be the result of the specific molecular structure of the insoluble lattice. We propose that RBs represent a general response of the cell to the accumulation of abundant, nondegradable protein(s) that fail to exit from the ER.

One hundred years ago, Russell described certain intracellular bodies in marginal areas of tumours, which he thought were fungi representing the etiological agent of cancer (Russell, 1890). The origin of these structures, which were later referred to as Russell bodies (RBs)[1], has been the subject of considerable research and controversy, over many years. They were thought by some to be pathological secretions or aggregates of normal secretion, or even secretory granules, while others proposed that they resulted from degeneration of plasma cells, from abortive differentiation of haemocytoblasts, or due to red blood corpuscles taken up by plasma cells. Pearse, in 1949, showed that they contained mucoproteins secreted by plasma cells (Pearse, 1949). Others confirmed these observations, and further showed the involvement of γ-globulins and antibodies (reviewed by Maldonado et al., 1966).

The origin of RBs has remained controversial. The involvement of the ER and a similarity between RBs and pancreatic secretory granules were suggested by Maldonado et al. (1966), who also suspected RBs to be similar in character to those described by Mott (1905). It is now generally agreed that "Mott" cells (or Morula cells) and "Russell" cells are essentially similar. The nature of this type of cells has remained under scrutiny. Interest revived with the observation that the "Mott phenotype" could be found in certain cell lines (Lockhorst et al., 1987) and also in hybridomas (Weiss et al., 1984; Alanen et al., 1985). The possibility that they are intermediate stages of normal secretion is no longer tenable, because hybridoma cells with RBs fail to secrete, or secrete very little Ig (Weiss et al., 1984; Alanen et al., 1985). The question which has been increasingly raised among those who considered RBs to be abnormal structures, is why they occur at all. The abnormality could originate from defects in the secretory apparatus, as suggested by Alanen et al. (1987). In this paper, we show that RBs can be generated by introducing abnormal Ig genes into otherwise healthy myeloma cell lines, and even in nonlymphoid secretory cells. We propose that RBs originate as an SOS compartment, where abnormal proteins which cannot be secreted, but have escaped intracellular degradation, can be accumulated without blocking the normal secretory pathway. These results imply the existence of subcompartments within the ER.

Materials and Methods

Cells, Plasmids, and Transfections

J558L (Oi et al., 1983), NSO (Cowan et al., 1974; Galfré and Milstein,

1. *Abbreviations used in this paper*: H, heavy chain; L, light chain; V, variable; C, constant; CH1, first constant domain of H; 2 ME, 2 mercaptoethanol; NP, (4 hydroxi 3 nitrophenyl)acetyl; RB, Russell bodies.

1981) C6 and C6λ (Cattaneo and Neuberger, 1987) were maintained in DME supplemented with 1 mM glutamine, penicillin, streptomycin, and 10% FCS, and were transfected by electroporation (Potter et al., 1984).

Plasmid pSV-V$\mu\Delta$CH1 (see Fig. 1), obtained by deleting the SacI fragments located between the enhancer region and the Cμ2 from pSV-Vμ1 (Neuberger, 1983), was a kind gift of Dr. M. S. Neuberger (MRC Laboratory of Molecular Biology, Cambridge, MA). To achieve Ig expression in nonlymphoid cells, the endogenous Ig promoter was substituted with the heat-inducible hsp70 promoter (Pelham, 1982). Thus, the exon encoding the first constant domain was deleted from pSV-HSVμ1 (a gift of Dr. J. Mason University of California at San Francisco, [Mason et al., 1988]) to generate pSV-HSV$\mu\Delta$CH1. To construct pSV-HSV$\mu_{ala}\Delta$CH1, the BglII-SacI vector fragment of pSV-HSV$\mu\Delta$CH1 was ligated to the Cμ2-4 fragment of pSV-Vμ[In 1,3,4,5]ala obtained by digestion with BglII (complete) and SacI (partial [Sitia et al., 1990]). pSV-Vλ1 was used to transfect NSO cells, while pSV-HSVλ1 was employed to generate nonlymphoid transfectants (Cattaneo and Neuberger, 1987). All heavy chain encoding plasmids carry the *gpt* gene, while those encoding light chains carry the *neo* gene.

Stable transfectants were selected in medium containing 5 μg/ml mycophenolic acid, 13.6 μg/ml hypoxanthine, and 0.25 mg/ml xanthine (all from Sigma Chemical Co., St. Louis, MO) for pSV2gpt, and in 1 mg/ml G418 (Gibco Laboratories, Grand Island, NY) for pSV2neo-based vectors. Positive clones were selected by immunofluorescence with class-specific antibodies as previously described (Sitia et al., 1987).

Biosynthetic Labeling, Immunoprecipitation, and Gel Electrophoresis

Cells were washed twice in methionine-free MEM and cultured for 2–18 h at 37°C in the same medium supplemented with 100–200 μCi/ml of [^{35}S]methionine (specific activity >800 Ci/mmol, Amersham, Milano, Italy), 2% dialyzed FCS, antibiotics, and 1 mM glutamine. Pulse-chase experiments were performed as previously described (Sitia et al., 1987, 1990). For sugar labeling, cells were cultured for 1 or 2 h in glucose-free DME supplemented with 2% dialyzed FCS and either [^{3}H]galactose (200 μCi/ml) or [^{14}C]mannose (50 μCi/ml, both from Amersham). Cells were then washed once in PBS and lysed in NP-40 (0.25%) unless otherwise indicated.

Lysates and supernatants of labeled cells were precleared with protein A-Sepharose (Pharmacia, Uppsala, Sweden) and immunoprecipitated with pure rabbit anti-mouse (RAM)-μ or monoclonal antiidiotypic antibodies Ac 38 (Reth et al., 1979) followed by protein A-Sepharose, or with sepharose-bound (4 hydroxi 3 nitrophenyl) acetyl (NP) (Reth et al., 1979; Neuberger, 1983). After washing three times in 500 mM NaCl, 50 mM TrisCl, pH 7.6, and 0.25% NP-40, and once in 5 mM TrisCl, NP precipitates were eluted in 100 mM free NP, whereas anti-μ or anti-idiotype immunoprecipitates were eluted at 95°C in 5% SDS. Immunoprecipitates were analyzed by reducing (Laemmli, 1970) and nonreducing (Ziegler and Hengartner, 1977) SDS-PAGE. Gels were fixed and impregnated with Amplify (Amersham) before exposure to Hyperfilm MP (Amersham) or Kodak X-Omat films at −80°C. For quantitative analyses, at least two exposures of the autoradiograms were scanned by an automated densitometer (LKB, Bromma, Sweden).

Western Blotting

Polyacrylamide gels were transferred electrophoretically to nitrocellulose paper (Schleier & Schuell, Dassel, West Germany). Filters were incubated for 1 h in PBS containing 3% milk powder (Gespal, Milan, Italy) and rinsed in PBS containing 0.05% Tween 20. To simultaneously detect heavy (H) and light (L) chains, filters were cut horizontally in correspondence of the prestained ovalbumin marker (46 kD), and the upper part, containing heavy chains, was incubated with alkaline phosphatase-conjugated rabbit anti-mouse-μ (Zymed, San Francisco, CA) and developed according to the manufacturer's instructions. A biotinylated goat anti-mouse λ, followed by peroxidase-streptavidin (both from Amersham), was used for the lower part of the filters, to detect L chains. A monoclonal rat anti-mouse BiP (Bole et al., 1986) was employed, followed by peroxidase-conjugated rabbit anti-IgG (Dakopatts, Glostrup, Denmark).

Immunofluorescence

Immunofluorescence was performed on poly-L-lysine-coated slides as described previously (Sitia et al., 1987). Fluoresceinated and rhodaminated goat anti-mouse-μ and goat anti-mouse-λ were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Rat anti-mouse BiP ([Bole et al., 1986] a gift of Dr. L. Hendershot, Memphis, TN) rabbit anti-mouse endoplasmin (a gift of Dr. G. Koch, Cambridge, UK) and rabbit anti-protein disulphide isomerase (from Dr. R. Freedman, Canterbury, UK) were used in indirect immunofluorescence followed by fluoresceinated goat anti-rabbit or goat anti-rabbit Ig (Cappell, Cochranville, PA). Fluoresceinated wheat germ agglutinin, a marker of the exocytic compartment downstream the Golgi apparatus (Tartakoff and Vassalli, 1983), was purchased from Sigma Chemical Co. and used according to the manufacturer instructions. Samples were inspected by either conventional or laser confocal microscopy (Leica CLSM, courtesy of A. DiFrancesco and A. Miani, Institute of Anatomy, University of Milan, Italy).

Cytochemistry and EM

Cells were fixed in suspension with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 30 min at room temperature. For cytochemical staining, fixed cells were washed with PBS, cytocentrifuged onto slides, and incubated with the substrates for 1 h at 37°C in the dark. The substrates for the localization of acid phosphatase and β-glucuronidase were prepared as previously described (Zicca et al., 1982; Ferrarini et al., 1980; Landay et al., 1984). For the ultrastructural studies, fixed cells were rinsed in cacodylate buffer, postfixed for 1 h with 1% osmium tetraoxide in the same buffer, dehydrated through a graded ethanol series, and embedded in Spurr's medium (Spurr, 1969). Thin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips 301 electron microscope.

Results

Induction of RB in Plasmacytomas Transfected with a Defective Heavy Chain

Plasmid pSV-V$\mu\Delta$CH1 (Fig. 1) encodes a μ chain that lacks the first constant domain (CH1). When this plasmid is transfected into the mouse myeloma J558L, which produces and secretes λ L chains, the transfected cell line (J[$\mu\Delta$CH1], see Table I), accumulates in its cytoplasm prominent RB-like structures (Fig. 2). Immunolocalization experiments showed that the defective H chain is primarily localized within these large vacuoles (Fig. 2, *a* and *c*). The structures are generally large and few in number (on a per cell basis), but cells with more than a dozen smaller vesicles are also detected. These structures are not found in J[μ1] cells, a J558L line harboring plasmid pSV-Vμ1 (Neuberger, 1983), which encodes wild type [wt]-μ (Sitia et al., 1987, 1990) or in other transfectants of J558L produced in our laboratory (not shown). The

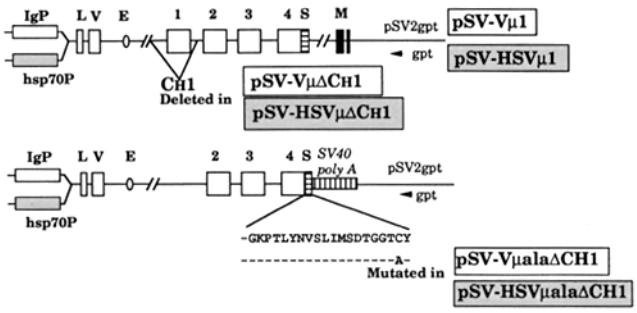

Figure 1. Structure of plasmids. The heavy chain-encoding plasmids are outlined. Construction of pSV-Vμ1 was described by Neuberger (1983). In pSV-HSVμ1 (Mason et al., 1988) the endogenous Ig promoter (*white box*) was replaced with the hsp70 promoter (*grey box*). Deletion of the first constant domain (*CH1*) was achieved by digestion with SacI and religation. The COOH-terminal cysteine was substituted with alanine by site-directed mutagenesis (Sitia et al., 1990). Exons are boxed, while the H chain enhancer is represented as a circle.

Table I. Properties of Cell Lines and Transfectants

Cell line-Plasmid	Cell type	Abbreviation
J558L [pSV-Vμ1]	mouse myeloma	J[μ1]
J555L [pSV-V$\mu\Delta$CH1]	($H^-\lambda^+$)*	J[$\mu\Delta$CH1]
J555L [pSV-V$\mu_{ala}\Delta$CH1]		J[$\mu_{ala}\Delta$CH1]
NSO [pSV-V$\mu\Delta$CH1]	mouse myeloma	N[$\mu\Delta$CH1]
NSO [pSV-V$\mu\Delta$CH1 + pSV-Vλ1]	(H^-L^-)*	N[$\mu\Delta$CH1-λ]
C6 [pSV-HSVμ1 + pSV-HSVλ1]	rat glioma‡	C6λ[μ1]
C6 [pSV-HSV$\mu\Delta$CH1 + pSV-HSVλ1]		C6λ[$\mu\Delta$CH1]
C6 [pSV-HSV$\mu_{ala}\Delta$CH1 + pSV-HSVλ1]		C6λ[$\mu_{ala}\Delta$CH1]

* Indicates endogenous immunoglobulin chain production; both J558L and NSO produce endogenous J chain.
‡ C6 glioma cells do not produce H, L, or J chains (Cattaneo and Neuberger, 1987).

vacuolar structures stain prominently also with anti-λ. However, unlike anti-μ, anti-λ stains brightly also other regions of the cytoplasm, corresponding to the ER and the Golgi apparatus (Fig. 2 *b*). Thus, in J[$\mu\Delta$CH1] cells, mutant μ chains are concentrated within RBs, but only a fraction of the L chains is diverted to them (see below). When the plasmid pSV-V$\mu\Delta$CH1 is used to transfect NSO myeloma cells, which do not express L chains, few if any RBs are detected in the transfectant (N[$\mu\Delta$CH1]; Fig. 2 *e*). The occasional vacuoles detected are much smaller than those found in J[$\mu\Delta$CH1]. Supertransfection of N[$\mu\Delta$CH1] with pSV-Vλ1 induces the formation of vacuoles (Fig. 2 *f*). Thus, the vacuole phenotype is not specific for one particular myeloma cell and is dependent on the protein structure, since L chains are essential.

At the EM level, the vacuoles appear to be delimited by a membrane covered by ribosomes, separated from a dense mass of material by a clear rim which excludes the electron dense dye (Fig. 3). Many cells containing vacuoles are mitotic (Fig. 3 *b*), indicating that the presence of these extremely dilated cisternae does not affect cell division. This is consistent with the observation of a similar doubling time in J[$\mu\Delta$CH1] and J[μ1] cells.

The presence of ribosomes on the cytosolic face of the membrane (see Fig. 3 *c* for a detail), suggests a relationship between the vacuoles and the ER. However, resident ER proteins are not concentrated in the vacuoles. Thus, antibodies against three such proteins, BiP (Fig. 2 *d*), protein disulphide isomerase, and endoplasmin (not shown), stained the vacuoles rather weakly (see also below). On the other hand, the staining pattern with fluoresceinated wheat germ agglutinin is indistinguishable in J[$\mu\Delta$CH1] and J[μ1] cells (Fig. 2, *g* and *h*, respectively), and more specifically we do not detect significant RB staining, suggesting that the protein has not traversed the Golgi apparatus.

RBs are often contiguous to normal ER stacks. Microscopical examination, however, does not reveal continuity between the two structures in myeloma transfectants. In addition, no fusion elements between endolysosomal vesicles and RBs are observed by EM or immunocytochemistry (data not shown).

Defective Secretion of Mutated Ig

The secretion of H and L chains differs considerably when the mutant J[$\mu\Delta$CH1] and the wild type J[μ1] transfectants are compared (Fig. 4 *a*). Very little mutant μ chain is precipitated from the supernatants of pulsed J[$\mu\Delta$CH1] cells even after prolonged chase periods (data not shown). The $\mu\Delta$CH1 chains present in the supernatant have a slower mobility on SDS-PAGE than the intracellular material, suggesting residual secretion rather than cell death. As for the L chains, it should be remembered that in secretory B cells L chains are present in large excess with respect to μ chains, and this may be even more marked in H chain transfectants. In the case of both J[$\mu\Delta$CH1] and J[μ1] the excess of L chains is at least 3 fold, and probably much larger (data not shown). The excess (free) L chains are secreted by J[$\mu\Delta$CH1] with kinetics similar to the control transfectants without RBs (Fig. 4 *a*). Therefore, the presence of RBs is compatible with protein secretion.

To further compare biosynthesis and secretion of mutant and wild type μ chains, equal numbers of J[$\mu\Delta$CH1] and J[μ1] cells were mixed, labeled for 2 h with [^{35}S]methionine, [^{14}C]mannose, or [^{3}H]galactose, and immunoprecipitated with anti-μ (Fig. 4 *b*). Under these conditions, no [^{35}S]methionine–labeled mutant H chain is detectable in the supernatant (Fig. 4 *b*, lane *2*). Less galactose is incorporated into $\mu\Delta$CH1 chains, as compared to wild type μ (Fig. 4 *b*, lanes *5* and *6*), but traces of secreted material can be detected in this way. The [^{14}C]mannose/[^{35}S]methionine ratio is approximately twice as high in J[$\mu\Delta$CH1] than in J[μ1], suggesting either increased incorporation or reduced trimming of mannose residues in mutant μ chains. The CH1 deletion involves one of the seven methionine residues as well as one of the seven potential N-linked glycosylation sites present in mouse μ chains; thus, it should have only marginal effects on quantitative labeling. Taken together, these data indicate that newly synthesized mutant IgM molecules are retained in a pre-Golgi compartment.

Assembly and Polymerization of Mutant IgM Are Essential for the Formation of RBs

The CH1 is the binding domain for both L chains and BiP (Bole et al., 1986; Hendershot et al., 1987). L chains are, however, capable of binding $\mu\Delta$CH1 chains through the VH domain in a noncovalent way. This is not the first example of a mutant Ig that lacks the CH1 domain but binds L chains (Secher et al., 1977). Under nonreducing conditions, the $\mu\Delta$CH1 bands comigrate in J[$\mu\Delta$CH1] and N[$\mu\Delta$CH1] excluding covalent interactions with λ (results not shown). That the deletion of CH1 does not affect VH-VL pairing is shown by

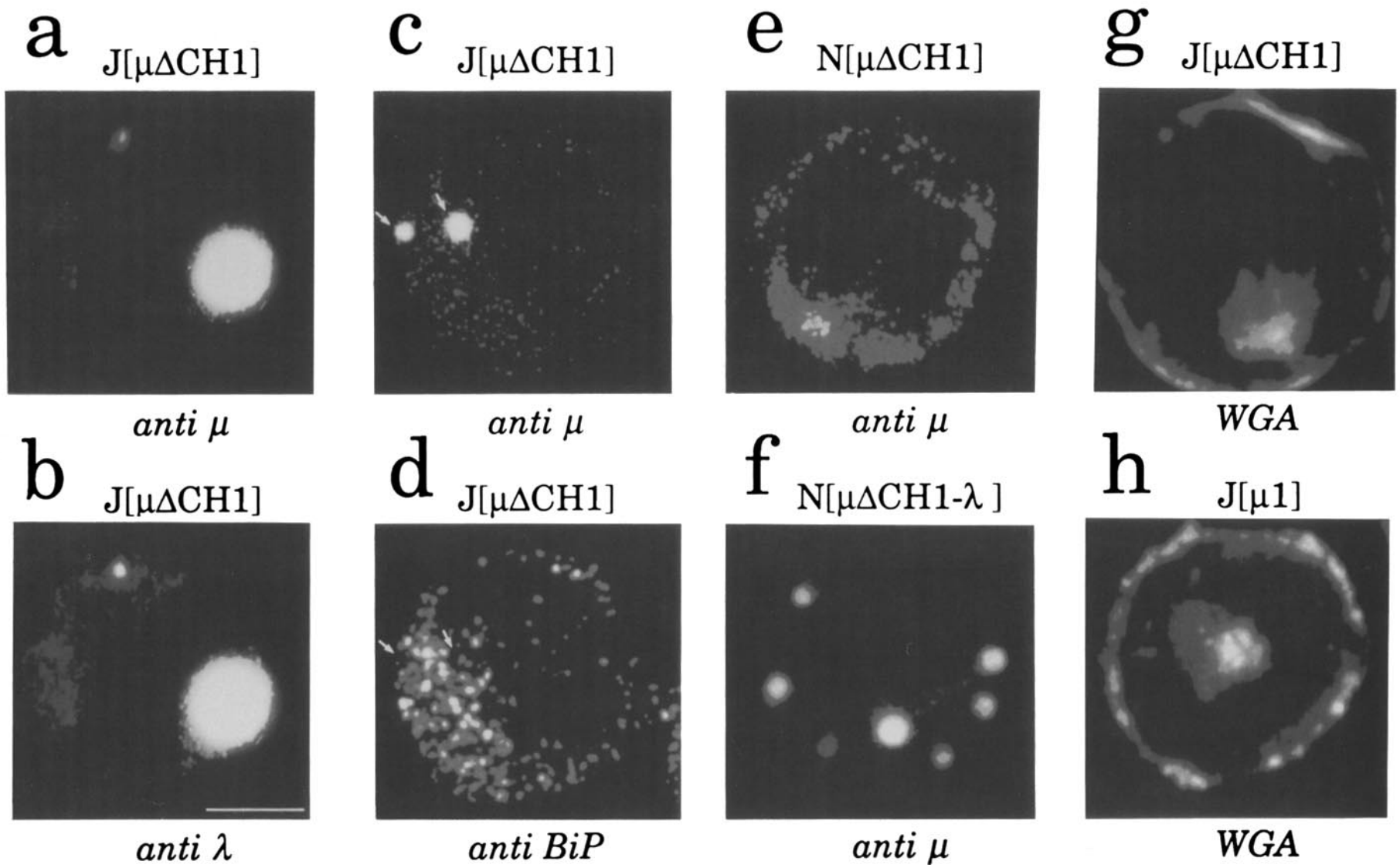

Figure 2. Structures resembling RB are present in J[μΔCH1] cells. Confocal immunofluorescence analysis of myeloma transfectants. (*a*, *b*, *c*, *d*, and *g*) J[μΔCH1]; (*e*) N[μΔCH1]; (*f*) N[μΔCH1-λ]; (*h*) J[μ1] cells. Slides were stained with fluoresceinated or rhodaminated goat anti-mouse-anti-μ (*a*, *e*, *f*, and *c*, respectively). *a* and *b* show the same cell stained simultaneously with fluoresceinated anti-μ (*a*) and rhodaminated goat anti-mouse-λ (*b*). Similarly, *c* and *d* show the same cell costained with rhodaminated anti-μ (*c*) and with rat anti-BiP followed by fluoresceinated goat anti-rat Ig (*d*). BiP appears to be largely excluded from the vacuoles (see *arrows*). In *g* and *h*, J[μΔCH1] and J[μ1] cells were stained with rhodaminated WGA. Bar, 5 μm.

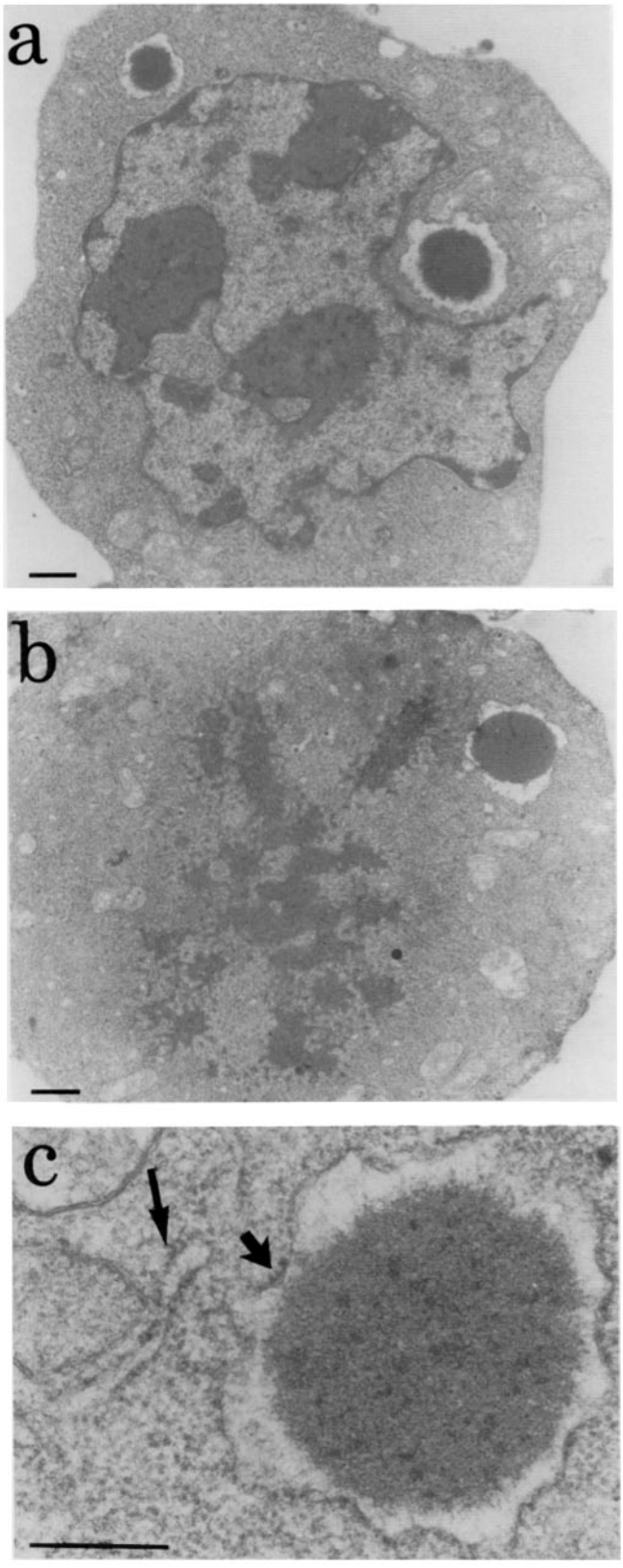

Figure 3. EM analysis of J[$\mu\Delta$CH1] cells. Note a mitotic cell in *b*, and, at higher magnification (*c*), numerous ribosomes on the membrane (cytosolic face) of the vacuole (*thick arrow*). Thin arrow points to a stack of normal ER. Bars, 1 μm.

precipitation of intracellular material with either the antigen (NP-Sepharose, Fig. 5 *a*, lane *2*) or the antiidiotypic antibody Ac38 (results not shown), both of which require VH-VL interaction (Reth et al., 1979). However, precipitation with anti-μ antiserum does not coprecipitate much L chain, which suggests that the interaction of L chain and $\mu\Delta$CH1 may be inhibited by the antiserum and/or that the noncovalent VH-VL association is stabilized by the antiidiotypic antibodies and by hapten. The higher ratio of L and H chain precipitated by antigen (see legend to Fig. 5 *a*) (and also by antiidiotype, not shown) suggests that the stoichiometry of the two chains is not the usual 1:1, but more likely 2:1 in favor of L chains. It remains to be seen whether this is a reflection of a real structural difference or an artifact due to variations in labeling resulting from different intracellular pools of unassembled chains.

The kinetics of accumulation of polymeric wild type and mutant IgM differ considerably. Thus, in the J[μ1] transfectant, as in other IgM secreting cells, an equilibrium between ~20% polymeric and 80% non-polymeric IgM is reached after between one and two hours of chase, when newly synthesized polymers are being secreted (Sitia et al., 1987). In the mutant J[$\mu\Delta$CH1], polymers are formed more slowly, and they continue to accumulate at the expense of preformed monomers (Fig. 5, *b* and *c*). Thus, after prolonged chase, labeled monomers are virtually undetectable, while polymers remain in abundance.

Mutant IgM Accumulates in RBs in the Form of Stable Aggregates

When the pellet of a lysate obtained with 0.25% NP-40 is extracted with 2% SDS, a considerable amount of $\mu\Delta$CH1 chains can be solubilized and detected by Western blotting (see Fig. 6 *a*, lane *6*). L chains are also detected in the NP-40–insoluble fraction. Mutant IgM is solubilized also by urea (8M, lane *7*) and, partially, by 2-mercaptoethanol (2ME, 700 mM, lane *9*). Analysis under nonreducing conditions (Fig. 6, lanes *10* and *11*), reveals that the IgM present in the insoluble material is largely in the polymeric form. On the contrary, in J[μ1] (Fig. 6, lanes *1–3*) as well as in other transfectants without RBs (not shown), virtually all Ig is found in the 0.25% NP-40–soluble fraction. Thus, part of $\mu\Delta$CH1 chains forms a precipitate that is held together by both covalent and noncovalent interactions. BiP (Fig. 6 *b*) and PDI (not shown) are found only in the soluble NP-40 fraction. Since resident proteins of the ER and excess light chains are excluded from the core precipitate containing mutant IgM, aggregation appears to be a specific self-assembly of a mutant Ig lattice. As shown by pulse-chase experiments (Fig. 6 *c*; see legend for experimental details), $\mu\Delta$CH1 chains are soluble in NP-40 immediately after synthesis and, in this form, subject to fast degradation (see Fig. 6 *d*). However, the undegraded remnant progressively accumulates within the insoluble fraction in the form of polymeric molecules. The precipitated material appears to be rather stable and after 21 h ~30% of the initial radioactivity is still detectable (Fig. 6 *d*). The slight reduction in intensity of the NP-40–insoluble band between 4.5 and 21 h of chase is matched by a proportional increase in the secreted material. This suggests that part of the precipitate may recycle into a soluble and secretable pool (see below). In the absence of L chains

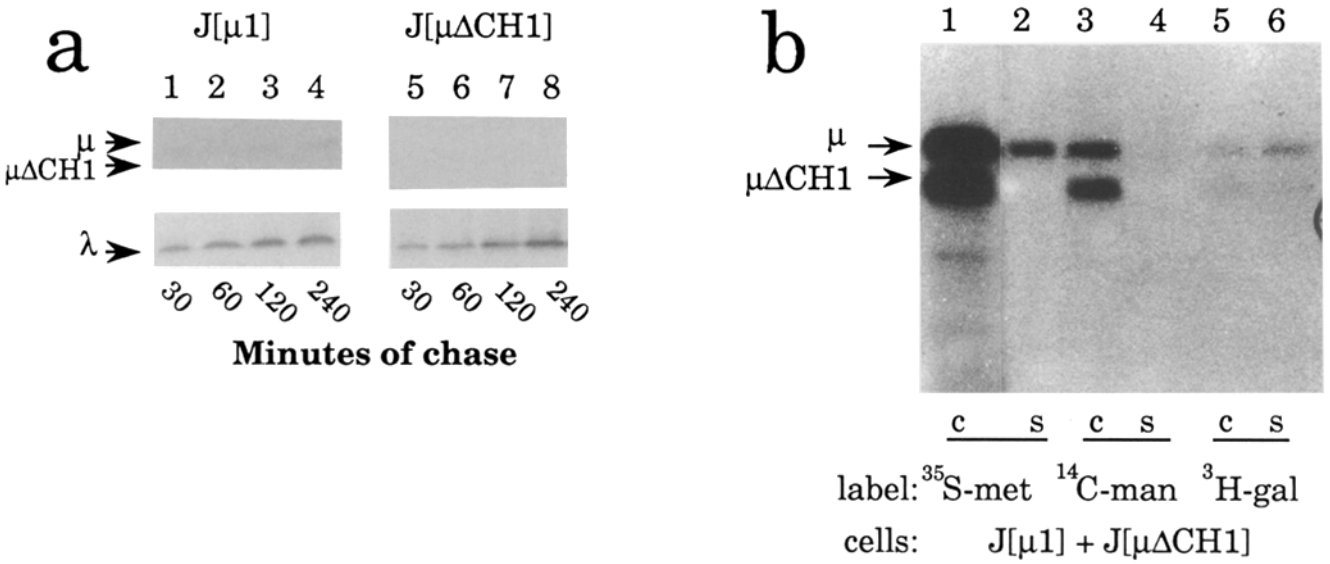

Figure 4. The secretory block in J[$\mu\Delta$CH1] is selective for mutant H chains. (*a*) J[μ1] (lanes *1–4*) and J[$\mu\Delta$CH1] (lanes *5–8*) cells were washed thoroughly and cultured at 1×10^6/ml in medium containing 1% FCS. At the indicated times, aliquots of the supernatants were harvested, concentrated fivefold, and analyzed by Western blotting with anti-μ (*upper panel*) and anti-λ (*lower panel*) antibodies. (*b*) Equal numbers of J[μ1] and J[$\mu\Delta$CH1] cells were mixed and labeled for 2 h with [^{35}S]methionine (lanes *1* and *2*), [^{14}C]mannose (lanes *3* and *4*) or [^{3}H]galactose (lanes *5* and *6*). Wild type μ and $\mu\Delta$CH1 chains differ in molecular weight, and can be easily identified on SDS-PAGE (see *arrows* on left hand margin). Cell lysates (*c*) and supernatants (s) were immunoprecipitated with anti-μ and analyzed by SDS-PAGE under reducing conditions. The μ1/$\mu\Delta$CH1 ratio (calculated by densitometric analyses of the autoradiogram) was 1.25 in the sample labeled with [^{35}S]methionine (lane *1*), and 0.65 when cells were labeled with [^{14}C]mannose (lane *3*).

(N[$\mu\Delta$CH1] cells) the mutant μ chain does not accumulate into an insoluble precipitate and is rapidly degraded (Fig. 6 *d*). That the insoluble material is likely to correspond to the content of RBs is indicated by the identification of RB-like structures in the 0.25% NP-40 pellets by immunofluorescence (not shown).

Mutating the COOH-terminal Cysteine Restores Secretion of $\mu\Delta$CH1 Chains

The importance of polymers in the formation of RBs is demonstrated by the phenotype of another cell line prepared by transfecting J558L with a mutant of $\mu\Delta$CH1 in which Cys 575 was replaced by Ala. The mutation restores efficient secretion (Fig. 7 *a*). NP-Sepharose precipitates $\mu_{ala}\Delta$CH1 chains, indicating that the latter are associated with λ. Like in the case of $\mu\Delta$CH1, this association is noncovalent. The transfectant cell line, J[$\mu_{ala}\Delta$CH1], contains few if any RBs, and anti-μ stains primarily the Golgi region (Fig. 7 *b*). Therefore, the two phenotypic characteristics of the Mott phenotype, impaired secretion and presence of RBs, are reversed by the removal of the COOH-terminal sulphydryl (SH) group. This SH group is responsible for both polymerization of IgM subunits and retention in the ER of assembly intermediates (Sitia et al., 1990).

$\mu\Delta$CH1 Chains Are Secreted in the Presence of Reducing Agents

It is known that 2ME induces secretion of unassembled, non-S-S–bonded intermediates (Alberini et al., 1990). Culturing J[$\mu\Delta$CH1] cells for 2 h in the presence of 2ME (7.1 mM) also induces secretion of $\mu\Delta$CH1 chains (Fig. 7 *c*). Since secreted $\mu\Delta$CH1 chains underwent terminal glycosylation (as indicated by their slower electrophoretic mobility) the result cannot be explained by reduced cell viability in the presence of reducing agents (see also Alberini et al., 1990). Thus, as in wild type μ chains, the COOH-terminal cysteine plays an important role in retention of unpolymerized chains, but unlike wild type chains, polymerization does not lead to secretion or to fast degradation, but to accumulation of polymers in RBs, likely in the form of insoluble precipitates. Interestingly, although less efficiently than when added immediately after the chase, 2ME can induce secretion of $\mu\Delta$CH1 chains even after overnight chase (Fig. 7 *c*), when virtually all mutant IgM is NP-40–insoluble within RB (Fig. 6 *c*). Thus, RBs are at least functionally connected with the secretory apparatus, and part of their contents can either continuously recycle between the RBs and the *cis*-Golgi or escape from the RBs, join the exocytic pathway, and be secreted.

Russell Body-like Structures Are Also Induced by the Defective Gene in Glioma Cells

The importance of the protein itself in the genesis of RBs is further emphasized by the appearance of similar structures in nonlymphoid cell transfected with pSV-HSV$\mu\Delta$CH1. The rat glioma cell chosen was C6λ which expresses a heat-inducible λ chain gene. In these cells, transfection with the wild type μ gene [μ1] allows expression and secretion of fully assembled IgM (Cattaneo and Neuberger, 1987). A similar construct was made, substituting the intact μ gene for $\mu\Delta$CH1 (driven by a heat-inducible promoter, see Fig. 1). Fig. 8 *a* shows that in the C6λ[$\mu\Delta$CH1] transfectant (but not in C6λ[μ1], Fig. 8 *b*) the defective H chain is prominently stained in intracellular structures. These structures are

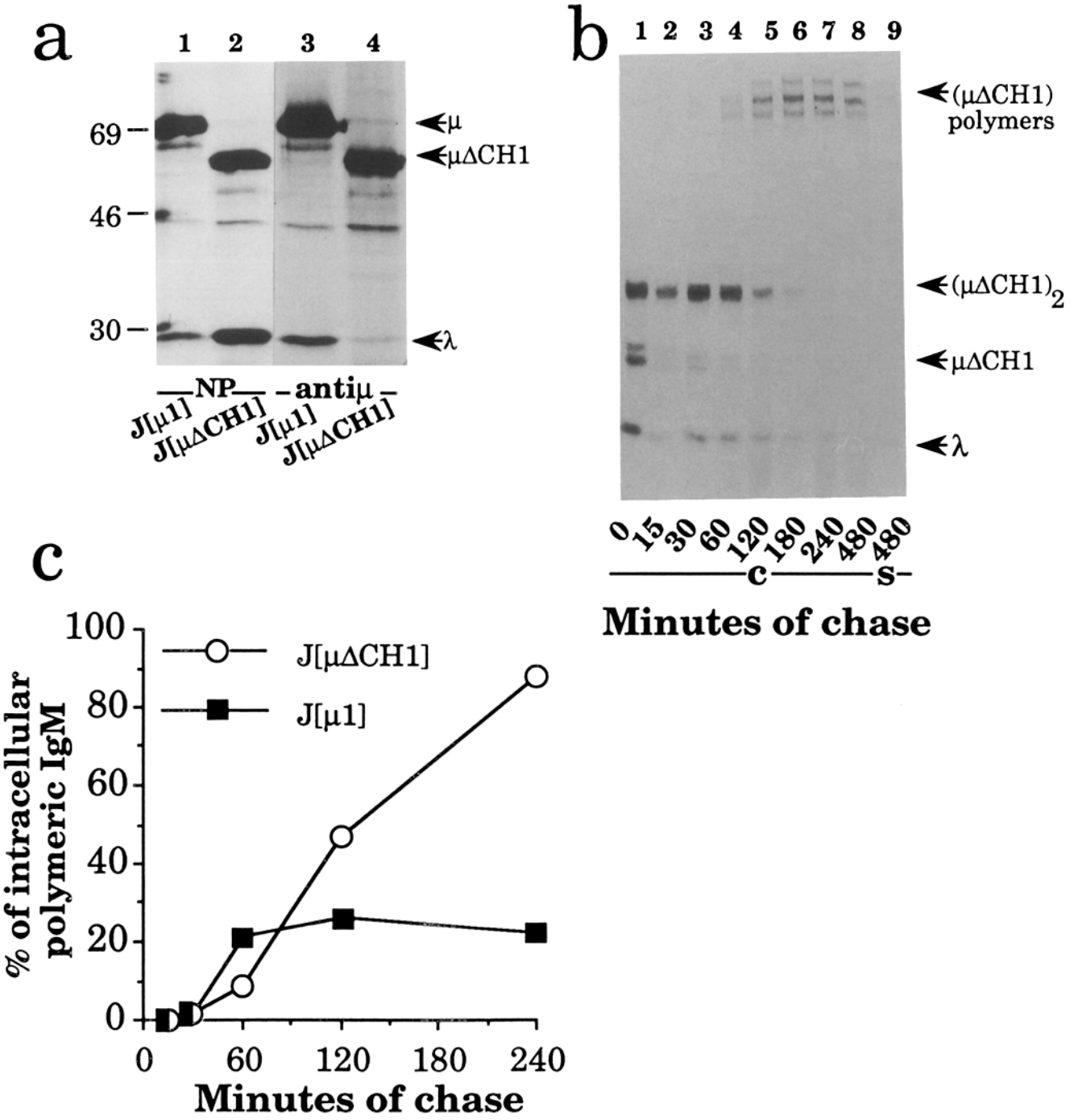

Figure 5. μΔCH1 chains assemble noncovalently with L chains and form polymers. (*a*) Noncovalent assembly between μΔCH1 and light chains. Lysates of J[μΔCH1] and J[μ1] cells labeled for 3 h with [^{35}S]methionine were immunoprecipitated with anti-μ antibodies and protein A-sepharose (anti-μ; lanes *3* and *4*) or with sepharose-bound nitrophenacetyl (*NP*; lanes *1* and *2*), and analyzed by SDS-PAGE under reducing conditions. The results with antiidiotypic antibodies (Ac38) were identical to those obtained with NP-Sepharose (not shown). In J[μΔCH1] cells, the H/L ratio, as calculated by densitometric analyses of the gel shown, was 44 in anti-μ precipitate (lane *4*) and 1.7 in NP-sepharose precipitate (lane *2*). By contrast, in J[μ1] cells the H/L ratio was 6.1 in both samples, as expected from a covalent interaction. (*b* and *c*) Rate of polymerization of mutant IgM. J[μΔCH1] cells were pulsed for 5 min with [^{35}S]methionine and chased for indicated times before lysis and immunoprecipitation with anti-μ. Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions (*b*). The rate of polymerization was calculated by densitometric analysis of the autoradiograms shown in *b* and compared to that of wild type μ (J[μ1] cells, data from Sitia et al., 1987). Data show the percentage of total intracellular radioactivity associated with polymers as a function of time (*c*). In J[μ1] cells, the plateau reflects the time at which the rate of polymerization of monomers (which are not secreted) and the rate of secretion of polymers reach an equilibrium. In J[μΔCH1], the ratio of polymers over monomers increases with time, because in this case polymers are not secreted.

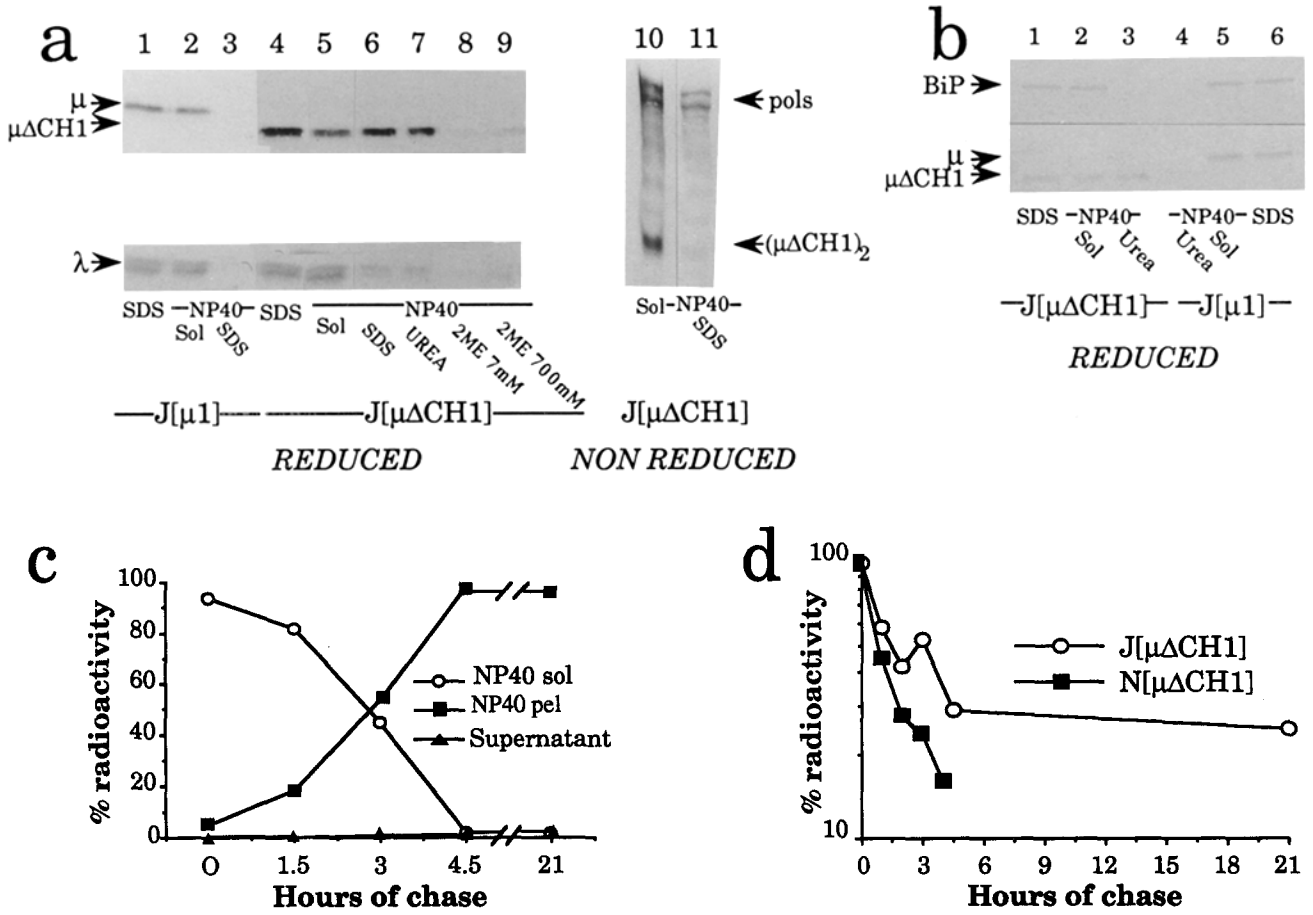

Figure 6. μΔCH1 chains form stable aggregates that accumulate in RBs. (*a*) Part of mutant IgM forms insoluble aggregates. Identical aliquots of J[μ1] and J[μΔCH1] cells were lysed with SDS or 0.25% NP-40 (all other lanes). The material soluble in SDS (lanes *1* and *4*) or NP-40 (*Sol*, lanes *2* and *5*) was run directly on SDS-PAGE, while the NP-40 pellets were extracted with 2% SDS (lanes *3*, *6*, and *11*), 8 M urea (lane *7*) or 2ME (7.1 mM, lane *8* or 710 mM, lane *9*). Lanes *10* and *11* were run on a 4% acrylamide gel under nonreducing conditions (Ziegler and Hengartner, 1977), and analyzed by western blotting with anti-μ. Lanes *1–9* were run under reducing conditions on a 10% acrylamide gel (Laemmli, 1970) and the blot was cut into two parts: the upper part was developed with anti-μ, the lower with anti-λ (see Materials and Methods). Prestained molecular weight markers (purchased from BioRad Italia, Segrate, Italy) were run to allow the identification of the bands as indicated by arrows. (*b*) ER resident proteins are excluded from the aggregates. Aliquots of J[μΔCH1] (lanes *1–3*) and J[μ1] (lanes *4–6*) were washed and lysed with SDS (lanes *1* and *6*) or NP-40. The NP-40–soluble material (lanes *2* and *5*) and the 8 M urea extracts from NP-40 pellets (lanes *3* and *4*) were resolved by SDS-PAGE under reducing conditions. Western blots were developed with rat anti-mouse BiP (*upper panel*), or anti-μ antibodies (*lower panel*). (*c*) Kinetics of accumulation of μΔCH1 chains into RBs. At the indicated chase times, J[μΔCH1] cells pulsed for 30 min with [^{35}S]methionine were lysed in 0.25% NP-40. Both NP-40–soluble material (*sol*) and the 8 M urea extract from pellets (*pel*, diluted 10-fold in PBS before immunoprecipitation), as well as the supernatants were immunoprecipitated with anti-μ and analyzed by SDS-PAGE and densitometry. The distribution of total recovered radioactivity between the different fractions (%) is shown at each point of chase. For total recovery, see data on *d*. (*d*) Kinetics of degradation of μΔCH1 chains in J558L or NSO transfectants. The intracellular turnover of μΔCH1 chains was determined by pulse–chase experiments of J[μΔCH1] and N[μΔCH1] followed by immunoprecipitation, SDS-PAGE, and densitometry. The results are expressed as percent radioactivity relative to 0 chase. Data represent the mean of two experiments.

smaller but more abundant than the ones formed in J558L, and in this sense more like classic RBs. When analyzed by EM, these structures show clear similarities with the vacuoles found in J[μΔCH1] (Fig. 8 *c*). Since C6 glioma cells do not synthesize endogenous J chains (Cattaneo and Neuberger, 1987), the latter are not necessary for RB formation. Removal of the COOH-terminal SH restores secretion of μΔCH1 chains also in glioma cells (Fig. 8 *d*): similarly, the presence of RB-like structures in C6 cells does not inhibit normal secretion (e.g., of endogenous fibronectin; Fig. 8, *upper arrow*).

Since the transfected μΔCH1 gene is driven by an inducible (heat shock) promoter, it was possible in this case to show that the formation of the RB-like vesicles was induced with the expected kinetics of accumulation of μΔCH1 (Fig. 8, and data not shown). Finally, formation of RB-like structures was largely prevented if heat-shocked C6λ[μΔCH1] were cultured in the presence of 2ME (data not shown).

Discussion

Biogenesis of Russell Bodies

Our data demonstrate that the synthesis of a mutated Ig (but

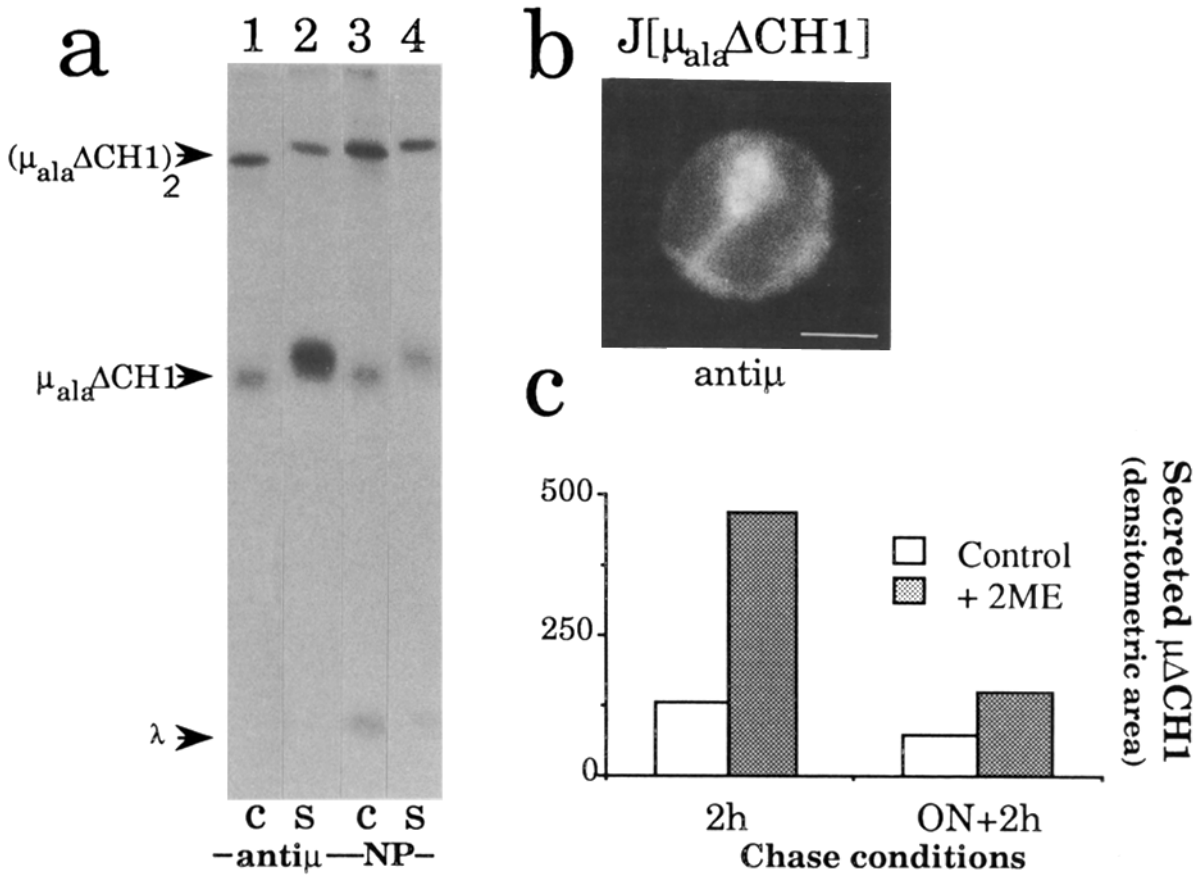

Figure 7. Substitution of the COOH-terminal cysteine and addition of reducing agents induce secretion of μΔCH1 chains. (*a*) Secretion of μΔCH1 lacking Cys575. Lysates (*c*) and supernatants (s) of J[μ_{ala}ΔCH1] cells labeled for 3 h with [^{35}S]methionine were immunoprecipitated with anti-μ (lanes *1* and *2*) or NP sepharose (lanes *3* and *4*) and analyzed by SDS-PAGE under nonreducing conditions. Arrows indicate migration of λ, μ_{ala}ΔCH1, and $(\mu_{ala}\Delta CH1)_2$. (*b*) Absence of RB-like structures in J[μalaΔCH1] cells. J[μ_{ala}ΔCH1] cells were stained with rhodaminated goat anti-μ. No vacuolar structures were detected, and anti-μ concentrates in the Golgi area. Sometimes, a punctuated staining, similar to N[μΔCH1], was observed. (*c*) Secretion of μΔCH1 is induced by reducing agents. J[μΔCH1] cells were pulsed for 30 min with [^{35}S]methionine, washed and chased for 2 h in the presence or absence of 7.1 mM 2ME either immediately after the pulse (2 h) or after overnight culture (ON+ 2 h). The anti-μ immunoprecipitates from the spent media were analyzed by SDS-PAGE and densitometry. Background precipitation (μ chain present in supernatant before the 2 h ±2ME) was subtracted. Bar, 5 μm.

not of the wild type counterpart) is sufficient to induce the formation of intracellular structures that share all the features of RBs. These structures were induced in the course of several independent transfections of J558L myeloma cells, which, as indicated by their capability of secreting endogenous λ chains and other transfected genes, have a functional secretory apparatus. The importance of the protein structure (as opposed to the cell phenotype, [Alanen et al., 1987]) in the induction of RBs, is underscored by the observation that transfection of the same mutant Ig into another myeloma line or into a nonlymphoid cell line also induces a similar phenotype. The morphology of the bodies was not identical. Paradoxically, the glioma transfectants contained the most commonly observed RB morphology, while in the myelomas RBs were larger and fewer in number (on a per cell basis).

Why do RBs form? There are several myelomas producing nonsecreted Ig chains or mutants that do not have the Mott phenotype (e.g., NS1 [Cowan et al., 1974; Galfré and Milstein, 1981], N[μl], J[γ2b-μtp] [Sitia et al., 1987, 1990]). Thus, the presence of a poorly or nonsecretable protein is necessary but not sufficient to induce the Mott phenotype. The essential difference, we suggest, is in the way such nonsecretable proteins are handled. It is most likely that the cell recognizes malfolded and unassembled polypeptides and quickly degrades them in the ER (Lippincot-Schwartz et al., 1988; Klausner and Sitia, 1990). The problem seems to arise when the ER fails to fully degrade structures that are otherwise unable to proceed through the secretory pathway, as is the case of the mutant IgM which we have studied. Formation of RBs might thus reflect the synthesis of abundant, nonsecretable molecules which, because of their relative stability, the cell has problems in disposing of.

Why is the mutant IgM not secreted? We have previously proposed that unassembled IgM subunits are retained in a pre-Golgi compartment by disulphide interchange reactions, and that formation of the intersubunit disulphide bonds releases the assembled IgM molecules, which can then proceed through the secretory pathway (Sitia et al., 1990; Alberini et al., 1990). In the case of the mutant, on the contrary, the formation of such bond seems to ultimately lead to the diversion of a stable aggregate into RBs. The critical role of this bond is demonstrated by the fact that substitution of Cys 575 for Ala allows efficient secretion of the double mutant. Reducing agents, albeit less efficiently, also induce

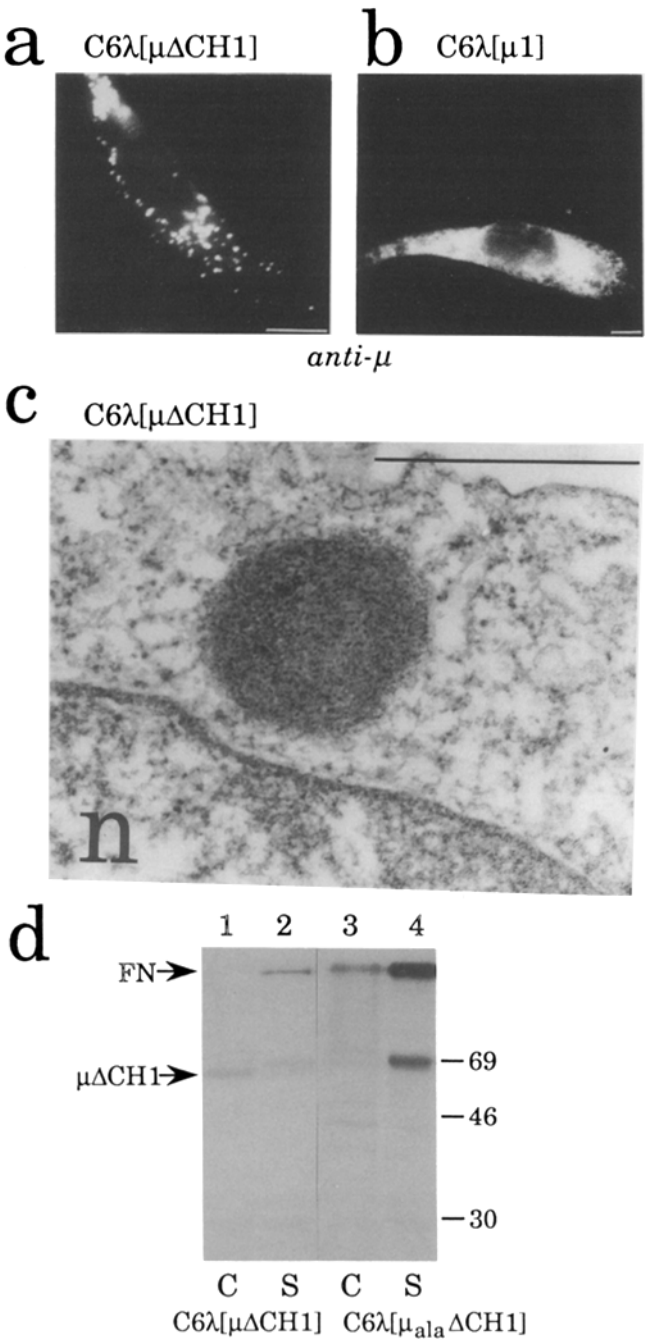

Figure 8. Secretion and intracellular localization of μ chain mutants in rat glioma cells. (*a–c*) RB-like structures in glioma transfectants. Immunofluorescence analysis of C6λ cells expressing μΔCH1 (*a*) or wt-μ (*b*) stained with fluorescent anti-μ after heat induction (1.5 h at 42°C and overnight at 37°C). EM (*c*) was performed on C6[μΔCH1λ] rat glioma transfectants after heat induction (2 h at 42°C followed by 3 h at 37°C). The figure depicts a RB-like structure. *n* = nucleus. (*d*) Defective secretion of μΔCH1 chains by glioma transfectants. C6λ cells expressing μΔCH1 (lanes *1* and *2*) or μ_{ala}ΔCH1 (lanes *3* and *4*) were labeled for 18 h with [^{35}S]methionine before immunoprecipitation of cell lysates (*c*) and supernatants (s) with anti-μ. Endogenous fibronectin (*FN*, see *arrow*) coprecipitates with our anti-μ antibodies. To optimize expression of the heat-inducible transgenes, cells were synchronized by culturing for 3 d in 1% FCS, transferred to 10% FCS for 5 h, and heat shocked for 2 h at 42°C before labeling. Bars: (*a*) 10 μm; (*b*) 10 μm; (*c*) 1 μm.

secretion of IgMΔCH1 by both lymphoid and nonlymphoid cells and prevent the formation of RB-like structures in glioma transfectants. The effect of 2ME may be that of preventing formation of polymeric structures while allowing secretion of intermediates.

Our interpretation of the contradictory role of Cys 575 is that the deletion of CH1 leads to noncovalent abnormal polymers. Deletion of the CH1 does not always lead to RBs, or to a total block of secretion (Kohler et al., 1982; Shulman et al., 1982). The specific structure of the VH and/or VL (and perhaps also the light chain isotype) must thus also play an important role in the formation of abnormal polymers. Such abnormal structures may occur because the hydrophobic phase of the CL domain normally interacts with the CH1 domain. In the absence of this interaction, CLs are likely to interact with each other, as they do in Bence Jones proteins (Milstein, 1965). It is therefore easy to envisage the formation of complexes which depart from the normal H_2L_2 configuration. This configuration is of the type (VHa-VLa) (VHb-VLb) (CLa-CHla) (CLb-CHlb), where a and b are used to symbolize different individual H-L pairs. In the mutant lacking CH1, CLa and CLb do not need to interact always with each other, but could instead interact with another L chain, which could be either free or already part of a different subunit. An abnormal H-L stoichiometry (see Fig. 5 *a*) would not thus be surprising. This would lead to complex mixtures of aggregates, some recognized as abnormal and degraded. Others however would retain the essential character of normally folded and assembled antibodies, since they bind antigen and interact with antiidiotypic antibodies, both requiring VH/VL pairing. The abnormal interactions may be similar to the one previously described in the mutant (IF2), an IgG lacking the CH1 domain (Secher et al., 1977). These abnormal structures, unlike the IgM mutants described here, are secreted, perhaps because they do not further polymerize to give rise to far larger insoluble aggregates. We thus suggest that small noncovalent aggregates form first, which are retained through Cys 575. When S-S bridges between subunits are formed, the resulting product is not a soluble pentamer which can be secreted, but an insoluble aggregate of polymers.

The Physiological Role of RBs

If the cell is to survive, it must be capable of handling such insoluble aggregates, which might otherwise block the secretory pathway. Segregating them into RB-like structures is a neat way plasmacells utilize to solve the problem. The fact that nonlymphoid cells are also capable of producing RBs, when faced with a similar problem, suggests that this may be a more general property of secretory cells. The high frequency of RBs in plasmacells may only reflect the high secretory capacity of these cells, coupled to the high frequency of spontaneous mutants within the Ig genes. In addition, lymphoid cells and myelomas are subjected to frequent microscopical examination. However, the problem of disposing of intracellular aggregates in secretory cells is not restricted to the immune system. Particularly relevant here are the recent studies of Tooze and coworkers on pancreatic cells (Tooze et al., 1989, 1990). They have shown that the previously described intracisternal granules (Palade, 1956) are composed of an insoluble aggregate of pancreatic enzymes

and proenzymes, held together by S-S bonds. Similarly, dilated ER cisternae, containing under-hydroxylated procollagen molecules, have been detected during chondrocyte in vitro differentiation. Addition of ascorbic acid, an essential cofactor in collagen hydroxylation, leads to the progressive disappearance of these structures (Tacchetti et al., 1987; Pacifici and Iozzo, 1988; Vertel et al., 1989). Similarities between intracisternal granules and RBs are easy to detect at the EM, but the differences are of particular interest. Images of intracisternal granules enclosed within autophagic vacuoles have been reported, and these organelles seem to be eventually disposed of by fusion with lysosomes (Tooze et al., 1990); by contrast, there is no evidence in the literature (nor so far in our experiments) that RBs become entrapped into phagophores and are eventually degraded by lysosomes. To provide an explanation for this difference, it may be mentioned that pancreatic cells do not divide at the speed of tumor or tissue culture cells, nor are they terminal cells like plasmacells. Thus, formation of RBs (and perhaps intracisternal granules) might lead to autophagic vacuoles only when cell division is not sufficient to avoid a constant accumulation incompatible with the cellular architecture.

Since the presence of RBs did not block secretion of L chains or fibronectin, sorting event(s) must occur within the ER which lead to efficient sequestration of the mutant IgM. The phenomenon may present some analogies with sorting events during regulated secretion, occurring however within Golgi cisternae (Kelly, 1985; Hashimoto et al., 1987; Wagner et al., 1991). Whatever the mechanisms implicated in sorting, the RBs appears molecularly distinct from the rest of the ER. Although at the moment we do not know whether this difference is restricted to the segregated contents, or concerns the limiting membranes as well, we feel that RBs should be regarded as subcompartments of the ER. It remains to be seen whether RBs correspond to a subregion of the ER present also in normal cells (but perhaps too small to be detected) or they are formed de novo in response to the presence of proteins with altered intracellular traffic.

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