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# Z-membranes: Artificial organelles for overexpressing recombinant integral membrane proteins

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**ABSTRACT** **We have expressed a fusion protein formed between the avian infectious bronchitis virus M protein and the bacterial enzyme β-glucuronidase in transgenic tobacco cells. Electron microscope images of such cells demonstrate that overexpression of this fusion protein gives rise to a type of endoplasmic reticulum membrane domain in which adjacent membranes become zippered together apparently as a consequence of the oligomerizing action of β-glucuronidase. These zippered (Z-) membranes lack markers of the endoplasmic reticulum (NADH cytochrome *c* reductase and ribosomes) and accumulate in the cells in the form of multilayered scroll-like structures (up to 2 μm in diameter; 20–50 per cell) without affecting plant growth. The discovery of Z-membranes has broad implications for biology and biotechnology in that they provide a means for accumulating large quantities of recombinant membrane proteins within discrete domains of native membranes.**

The secretory pathway of eukaryotic cells comprises the endoplasmic reticulum (ER), Golgi apparatus, plasma membrane/cell surface, lysosome (vacuole), and various endosomal and storage compartments. The mechanisms regulating the movement of proteins through this pathway have been intensively investigated in mammalian (1, 2), yeast (3), and, to a lesser extent, in plant (4) cells. Progress in elucidating the molecular mechanisms of protein trafficking through the plant Golgi has been hampered by the observation that its structure and function vary between different tissue types (5) and by a lack of resident protein probes and well-defined mutants as available in mammalian and yeast cell systems (6). We have been exploring the possibility of using heterologous proteins, such as the coronavirus avian infectious bronchitis virus (IBV) M protein, to probe the functional organization of the plant Golgi apparatus. In animal cells, IBV assembles intracellularly by budding into Golgi cisternae from whence the virions are transported to the cell surface (7). Localization of the assembly process to cis-Golgi cisternae appears to involve the IBV M protein, which when expressed alone is targeted to cis-Golgi cisternae (8). IBV M protein is an integral membrane glycoprotein with a molecular mass of 25–33 kDa depending on its glycosylation (9). A short luminal N terminus, carrying two sites for N-linked glycosylation, is followed by three transmembrane domains and a large cytoplasmic C terminus (8, 9). Targeting of IBV M protein to cis-Golgi cisternae is mediated by the first transmembrane domain (9, 10).

To simplify the detection of the IBV M protein in plant cells, we constructed a chimeric gene encoding a fusion protein between the IBV M protein and the 73-kDa bacterial enzyme β-glucuronidase (GUS), whose enzymatic activity is commonly used to monitor gene expression in transgenic plants (11, 12). We found, unexpectedly, that expression of the chimeric gene in tobacco cells leads to the formation of a novel membrane organelle. The IBV M–GUS fusion protein becomes trapped within specialized, zippered ER membrane regions, most likely due to the oligomerization of the GUS domains of fusion proteins in adjacent membranes. As a consequence of this zippering process, the chimeric proteins are sequestered away from the rest of the components of the ER, which allows them to accumulate in a stable and presumably active form.

## MATERIALS AND METHODS

**Recombinant Constructions.** The IBV M coding region was amplified via PCR, using pSV/IBV E1 as the template (8) and synthetic primers (5′-primer, 5′-GCG CGT CGA CCG ACC ATG TCC AAG GAG ACA AAT-3′; 3′-primer, 5′-GGC CCC CAT GGT GTA AAG ACT ACT TCC-3′). This eliminates the IBV M stop codon and provides unique 5′ *Sal* I and 3′ *Nco* I sites. Next, the 1.8-kb *Hin*dIII/*Eco*RI fragment from pRAJ 275 (Clontech) containing the GUS coding sequence was cloned into *Hin*dIII/*Eco*RI-cut pJIT 117 (13) to form pBAP 15. This has unique *Hin*dIII, *Sal* I, and *Nco* I sites located between the cauliflower mosaic virus 35S promoter and the GUS coding region. The *Sal* I and *Nco* I sites were separated by insertion into *Hin*dIII/*Sal* I-cut pBAP 15 of the 16-bp polylinker fragment excised from pBlueScript SK(−) by *Hin*d-III and *Xho* I, to form plasmid pFG 10. The *Sal* I/*Nco* I-cut PCR product of the IBV M coding sequence was then inserted into the corresponding sites in pFG 10 to form pFG 11A. The 4.0-kb *Kpn* I fragment from pFG 11A was finally transferred into *Kpn* I-cut pBIN 19 (14) to form pFG 14.

**Production of Transgenic Plants.** Agrobacterium-mediated transformation was employed to transfer pFG 14 into tobacco (15). Of the transgenic plants expressing GUS activity, plant pFG 14-23 was maintained for analysis and generation of suspension cultures (12).

**Protoplast Isolation and Transfection.** Protoplasts were isolated and transfected with pFG 11A and pBAP 15 as described (12).

**Homogenization and Fractionation Procedures.** Transfected protoplasts or transgenic leaf tissues were homogenized in 1× GUS extraction buffer (11) for GUS assay, in 2× SDS/PAGE loading buffer (16) for gel electrophoresis, or in HB-S medium (17) for subcellular fractionation. Differential centrifugation was employed to separate homogenates into fractions designated nuclei, microsomes, and cytoplasm as described (16). Microsomal fractions were fractionated on 20–50% linear sucrose gradients as described (16). The fractions were assayed for the activities of GUS and of various marker enzymes and for sucrose concentration (11, 12, 16).

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Abbreviations: ER, endoplasmic reticulum; GUS, β-glucuronidase; IBV, infectious bronchitis virus; Z-membrane, zippered membrane.

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**Gel Electrophoresis and Western Blotting.** Proteins were separated using one-dimensional SDS/PAGE gels, transferred onto membranes, and probed with polyclonal rabbit anti-GUS IgG (1:1000; Molecular Probes) and goat anti-rabbit IgG (heavy plus light chain) alkaline phosphatase conjugate (1:10,000; Sigma) as described (16).

**Immunofluorescence and Electron Microscopy.** Suspension culture cells were fixed with 2% paraformaldehyde in K medium (16) for 1 hr and treated with 1% (wt/vol) cellulysin and 0.2% (wt/vol) macerase in K medium for 2 hr at 25°C. The cells were permeabilized in TSW medium (16) for 30 min and incubated in darkness with rabbit anti-GUS IgG (1:100), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch) in TSW lacking SDS (16). The cells were examined using a Zeiss Axioskop fluorescence microscope. Electron and immunoelectron microscopy was done as described (18).

## RESULTS

**Characterization of the IBV M–GUS Fusion Protein.** Whereas expression of the native IBV M protein was not detected in transfected protoplasts and transgenic plants, expression of the IBV M–GUS fusion protein could be readily observed, either via gel electrophoresis or via enzymatic assay of GUS activity (Fig. 1). The fusion protein has an estimated molecular mass of 95 kDa (Fig. 1 *A* and *B*), which is very close to the size predicted by its primary sequence, and is consistent with a lack of glycosylation. Tunicamycin treatment *in vivo* and endoglycosidase H treatment of *in vitro* extracts did not alter the mobility of the fusion protein (data not shown). Twin protein bands were observed in extracts from transgenic plants (Fig. 1*B*) and suspension culture cells (data not shown), whereas only one larger band was observed in extracts from transfected protoplasts (Fig. 1*A*). The reason for this is unknown. The fusion protein behaved as if intrinsic to membranes; detergent treatment, but not high salt, solubilized GUS activities from microsomal fractions isolated from transgenic plant leaves (Fig. 1*C*). This suggests that the signal sequence associated with the N terminus of the IBV M protein is functional in plants, specifying cotranslational insertion into the ER membrane.

**Gradient Analyses of Endomembrane Fractions Carrying the IBV M–GUS Protein.** To determine which compartment within the secretory pathway houses the fusion protein, protoplasts were analyzed at various times after transfection. When analyzed 24 hr after transfection, the membranes carrying GUS activity (Fig. 2*C*) were distinct from those containing chlorophyll (Fig. 2*A*), the ER marker NADH cytochrome *c* reductase (Fig. 2*B*), and the plasma membrane marker vanadate-sensitive ATPase (Fig. 2*E*), but overlapped with membranes carrying latent inosine diphosphatase, a marker for the Golgi (Fig. 2*D*). At the earliest time points after transfection, GUS activity was solely detected in the membranes of the ER (Fig. 2*C*) but progressively accumulated within membrane fractions having higher densities (Fig. 2*C*). The steady-state location of accumulation of GUS activity was determined through analysis of transgenic plants constitutively expressing IBV M–GUS. The peak of GUS activity was broad and similar in position to that seen 24 hr after transfection but lacked a separate peak coincident with the ER (data not shown). This is consistent with the idea that the recombinant integral membrane protein is synthesized on ER-bound polyribosomes and cotranslationally inserted into the ER membranes but then accumulates within a membrane compartment of higher density than the ER.

**Induction of Novel Subcellular Structures by Expression of the IBV M–GUS Protein.** Electron microscopy of suspension culture cells expressing the fusion protein revealed membranous structures not found in nontransformed control cells (Fig. 3). These structures, termed zippered membranes, or Z-

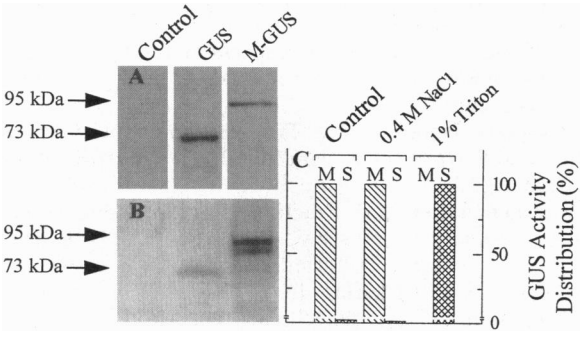


FIG. 1. Expression and characterization of IBV M–GUS in transfected protoplasts and transgenic plants. (*A* and *B*) Western blotting of transfected protoplasts (*A*) and transgenic plants (*B*) expressing null (Control), the native GUS and IBV M–GUS proteins. The viable protoplasts (20 hr after transfection) and the isolated microsomal and cytosolic fractions from the transgenic leaves were used for the respective analyses. (*C*) Extractability of IBV M–GUS from membranes. Microsomes from pFG 14-23 leaves (2 mg of protein) were incubated in HB-S medium (Control), HB-S medium with 0.4 M NaCl, and with 1% Triton X-100, respectively, at 22°C for 30 min. Microsomes (M) and incubation media (S) were then collected for GUS assay after centrifugation.

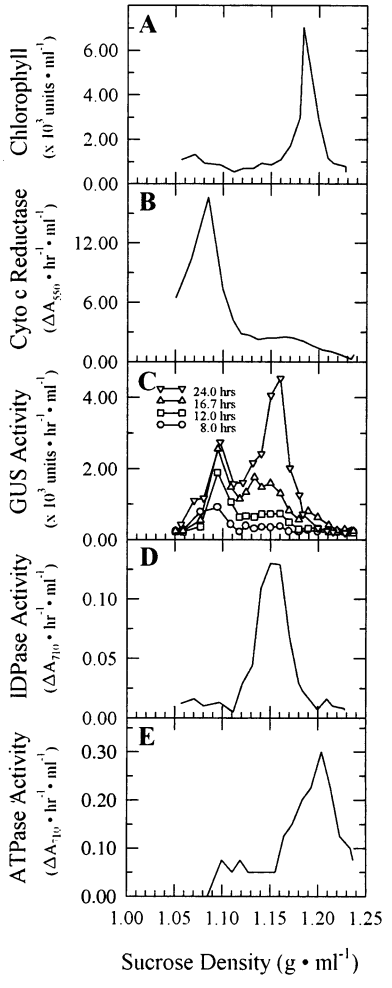


FIG. 2. Early biosynthesis and accumulation of IBV M–GUS in the plant secretory pathway. Viable protoplasts ($10^6$), purified 8, 12, 16.7, and 24 hr after transfection, were analyzed on 20–50% linear sucrose gradients. IDPase, inosine diphosphatase.

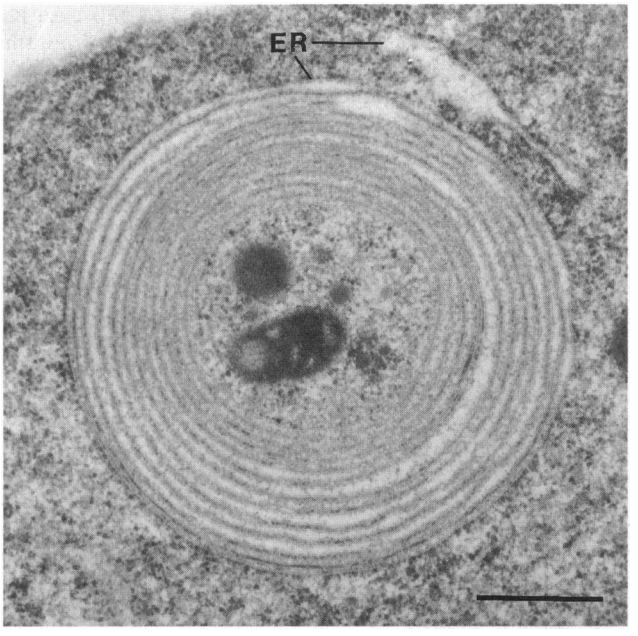


FIG. 3. Electron micrograph of a cross-sectioned Z-membrane whorl and associated ER membranes within a pFG 14-23 suspension cultured tobacco cell. (Bar = 0.5 μm.)

membranes, appear either as appressed, parallel membrane sheets or as whorl-like structures consisting of tightly wrapped cisternae in a concentric or spirillar configuration (Fig. 3) and appear to be continuous with the cisternae of the rough ER (Fig. 4*A*). Densely stained material is associated with the cytoplasmic surfaces of the appressed membranes, but the bound ribosomes are excluded (Figs. 3 and 4 *A* and *B*). The luminal spaces are comparable in width and staining to those of conventional rough ER. The GUS epitope is associated with the densely staining material at the cytoplasmic interface of the adhering Z-membrane cisternae (Figs. 3 and 4). This localization is consistent with the predicted orientation of the IBV M–GUS fusion protein, in which the GUS domain is exposed to the cytoplasmic surface of the ER cisternae. Neither the nonappressed, conventional rough and smooth ER membranes nor the Golgi stacks were labeled by the anti-GUS antibodies, nor was labeling observed over the cytosol (data not shown). Thus, virtually all IBV M–GUS fusion proteins are incorporated into Z-membrane structures, which apparently permit the nonaltered ER and Golgi cisternae to continue normal functions.

**Size and Distribution of Z-Membranes.** The number of cisternae wrapped in the Z-membrane whorls varied from 2 to >10; the overall diameter of the whorls ranged from 1.5 to 3 μm. Indirect immunofluorescence labeling of the transformed cells revealed numerous bright spots of fluorescence throughout the cytoplasm (Fig. 5). The size and the distribution of these spots match the size and the distribution of Z-membranes seen in the electron micrographs. From these data (Fig. 5), we estimate that the transformed cells contained 20–50 Z-membranes, constituting a total membrane surface area of about 500 $\mu m^2$ per cell. Z-membrane whorls of similar structure were also observed and labeled in leaf mesophyll cells of the transgenic plants (data not shown).

**Mechanism of Z-Membrane Formation.** A mechanism for the formation of Z-membranes can be devised based on the predicted topology of the chimeric IBV M–GUS protein coupled to the observation that the enzymatically active form of GUS is a homotetramer. In this model, the anchoring of the GUS domain to the cytoplasmic face of the ER mediated by the transmembrane domains of IBV M facilitates zippering of adjacent membranes. Progressive zippering would align newly

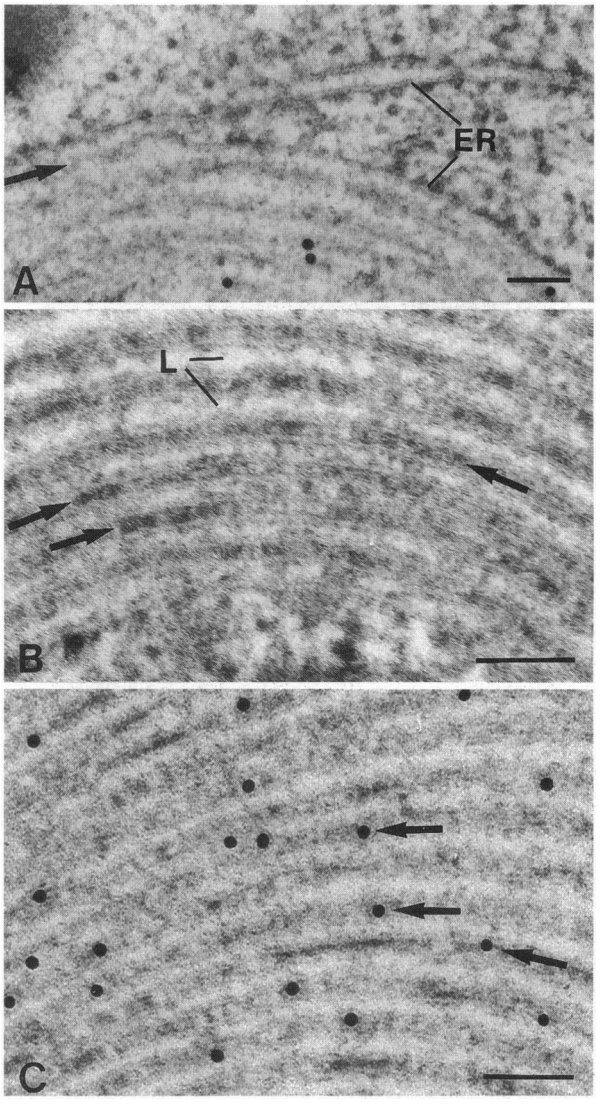


FIG. 4. Electron and immunoelectron micrographs of cross-sectioned Z-membranes. (*A*) Transitional region between nonappressed rough ER membranes and a GUS-zippered membrane domain (arrow). (*B*) GUS-zippered membrane domain showing substructure of the zippered membrane surfaces (arrows). L = ER lumen. (*C*) Z-membranes immunolabeled with anti-GUS antibodies. Note that most of the gold labels (arrows) are over the more darkly staining zippered membrane structures. (Bars = 0.1 μm.)

synthesized IBV M–GUS monomers, thereby promoting further zippering, ultimately leading to spontaneous self-assembly of ordered membrane structures, in which the self-sequestered protein component largely comprised IBV M–GUS. To test this mechanism, protoplasts were transfected separately with pBAP 15 (encoding the native cytoplasmic form of GUS), with pFG11A, or with an equimolar mixture of the plasmids. The intact protoplasts and their microsomes were analyzed via SDS/PAGE and Western blotting. pBAP 15-transfected protoplasts contained native GUS, but this was not found in the microsomes, as would be expected based on its cytoplasmic location (Fig. 6). Protoplasts and microsomes from protoplasts transfected with pFG11A contained a single GUS isoform, having a mass appropriate for the chimeric IBV M–GUS molecule (Fig. 6). Protoplasts and microsomes from protoplasts cotransfected with the two plasmids contained two GUS isoforms; one was identical in mass to the IBV M–GUS chimeric molecule, and the other was identical in mass to the native GUS molecule. Recovery of the cytosolic form of native

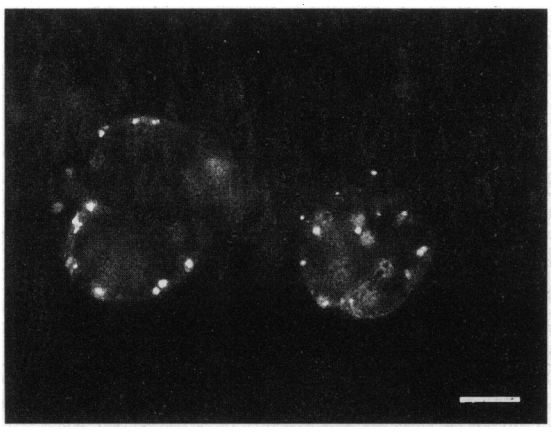

FIG. 5. Indirect immunofluorescence labeling of suspension cultured cells expressing IBV M–GUS.

GUS in association with the microsomes of cotransfected protoplasts is consistent with the formation of heterooligomers between GUS and membrane-bound IBV M–GUS, as predicted by the hypothesis.

## DISCUSSION

Under normal circumstances, integral membrane proteins entering the secretory pathway become anchored in the ER membrane in various configurations (19). After co- and post-translational modifications, only properly folded and assembled proteins can exit the ER and are then transported anterogradely to further compartments under the general aegis of default secretion (1, 2). Misfolded proteins are retained in the ER and are subsequently degraded (1, 2). It has been found that many recombinant proteins are vulnerable to trapping and degradation within the secretory pathway, particularly in experiments involving the study of protein trafficking (20). In the case described here, a contrary behavior is seen: the IBV M–GUS fusion protein, although trapped within the ER, appears to escape degradation and becomes concentrated in discrete subdomains of the ER, the Z-membrane domains. We postulate that GUS oligomerization-induced zippering of the IBV M–GUS containing membranes is responsible both for preventing the fusion proteins from exiting the ER through transport vesicles and for excluding other ER resident proteins, including proteases, from the Z-membrane domains. Proof that oligomerization of the GUS domains occurs in the Z-membranes is provided by the observation of GUS enzyme activity (Figs. 1*C* and 2*C*), which requires GUS tetramers. The demonstration that cytosolic native GUS can form heterooligomers with microsomal IBV M–GUS (Fig. 6) confirms the predicted membrane topology of the IBV M–GUS molecule and provides strong evidence that the zippering process involves GUS oligomerization. Furthermore, in anti-GUS immunolabeling experiments, only the

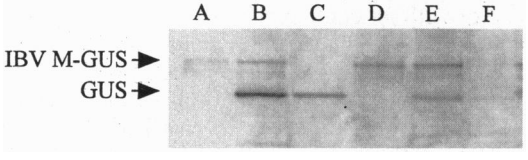


FIG. 6. Analysis of heterooligomer formation between the native cytosolic GUS and the membrane intrinsic IBV M–GUS, using SDS/PAGE and Western blotting. Lanes A–C were loaded with whole protoplast samples expressing IBV M–GUS alone, IBV M–GUS and GUS cotranslationally, and GUS alone, respectively. Lanes D–F were loaded with the microsomal fractions isolated from the protoplasts employed in lanes A–C, respectively. Protein amounts corresponding to equal numbers of protoplasts were loaded in each lane.

Z-membrane domains become labeled (Fig. 4 and data not shown). The exclusion of resident ER proteins from the Z-membranes is indicated by the lack of ER marker enzymes from the main Z-membrane fraction (Figs. 2*C*) and by the exclusion of bound polyribosomes (Figs. 3 and 4). Thus, Z-membranes are specialized ER compartments within which IBV M–GUS fusion protein becomes highly enriched.

The formation of Z-membranes in transgenic cells highlights both the perils of using fusion proteins with reporter domains for localizing proteins in transgenic cells and the potential of using oligomerizable proteins such as GUS in fusion protein constructs to accumulate desired proteins in transgenic organisms. Production of foreign proteins within transgenic hosts is often limited by their instability, their tendency to form insoluble aggregates, their loss of activity, and/or their toxicity to the host. This is particularly true for the overexpression of integral membrane proteins. Z-membranes offer a solution to these problems by sequestering foreign proteins, immediately after biosynthesis, away from the remainder of the cellular contents. This limits the exposure of these proteins to degradative enzymes, potentially preserves their activity, and greatly limits their ability to interfere with normal host functions.

Based on our studies to date, the criteria for producing engineered proteins with the potential to form Z-membranes are a membrane insertion signal, one or more transmembrane domains, and a cytoplasmic or luminal domain with a strong propensity to oligomerize. In addition, the oligomerized structures must be large enough to prevent entry into transport vesicles. This concept is applicable to all transformable eukaryotic cells. In our case, of 19 independently transformed plants, 17 plants stably and constitutively express the active IBV M–GUS fusion protein without visible deleterious effects. We have also successfully produced Z-membranes in yeast (21), and N. Raikhel (Michigan State University; personal communication) has recently found that organelles similar to Z-membranes accumulate in tobacco plants expressing an ERD2 (KDEL receptor)–GUS fusion protein. We therefore postulate that the Z-membrane system is potentially applicable as a general method for overexpressing membrane proteins in transgenic cells.

The ability of membrane systems, and particularly the ER, to adjust to major changes in protein contents is well-documented; examples include the accumulation of detoxifying enzymes in response to lipophilic drugs such as phenobarbitol (22), the overexpression of native membrane enzymes through the experimental inhibition of enzyme activities (23), and the overexpression of transgenes (24, 25). In some instances, the overexpression of native ER proteins has been shown to produce novel intracellular membrane structures (24–26). However, the mechanisms governing the formation of these special membrane compartments are still poorly understood, which limits their use as tools for overexpressing foreign proteins in transgenic cells. This contrasts with the Z-membrane system in which the membrane-zippering function and the segregation of the membrane proteins appear to be based on the native ability of GUS to form tetrameric aggregates (Fig. 7).

The discovery of the artificial Z-membrane organelle and its underlying mechanism of formation has broad implications for biological research and biotechnology. Aside from affirming the concept of "kin recognition" as a mechanism for retaining resident proteins in specific membrane compartments (27), it provides researchers with a new tool for overexpressing recombinant membrane proteins in transgenic cells. Possible uses include, but are not limited to, overexpressing membrane proteins for biochemical and crystallographic studies, membrane enzymes for processing of lipidic molecules (28), antigens for oral vaccines (29), and sensory molecules for novel detection systems (30, 31).

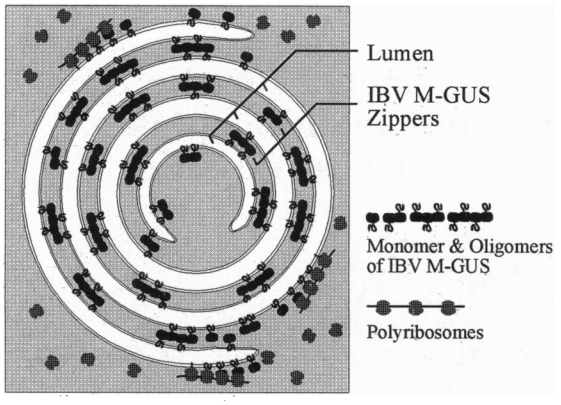


FIG. 7. Diagram illustrating how the postulated oligomerization of the GUS domains of the IBV M–GUS fusion proteins could produce the multilayered Z-membrane whorls shown in Figs. 3 and 4.

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