Membrane-bound immunoglobulin (mlg) is the antigen receptor on B lymphocytes mediating early events in antigen presentation and signal transduction. Wild-type human mlgM constructs transfected into the murine B-cell lymphoma A20 are expressed as transmembrane proteins with antigen presentation and signaling functions comparable to the endogenous mlgM2A; the transfected wild-type mlgM is internalized rapidly after anti-Ig cross-linking. Transfected constructs lacking the normal three-amino acid cytoplasmic tail are expressed exclusively as phosphatidylinositol-linked proteins, lack both antigen presentation and signal transduction functions, and are internalized slowly following anti-Ig binding. The molecular mass of the cytoplasmic tail-deleted phosphatidylinositol-linked Ig molecule is consistent with cleavage of the transmembrane residues during processing. Cytoplasmic domains may therefore regulate the mode of expression of membrane proteins and thereby influence their functional capabilities.

Cell surface proteins may be expressed either as integral transmembrane proteins or as molecules covalently linked to phosphatidylinositol (PI)1 (1, 2); examples of the latter (reviewed in Refs. 1 and 2) include intercellular adhesion molecules (e.g. LFA-3 in lymphoid cells (13)) and glycoproteins that may transduce lymphocyte-activating signals (e.g. Thy-1 (4) and Ly-6 (5)). Although the functional significance of PI-linked versus transmembrane protein anchors is unknown, PI linkage confers an increased sensitivity to release by specific phospholipases and subsequent degradation (1). The structural features of a protein that determine in which form it will be expressed are also not well defined, and the same molecule may be present in both forms (e.g. LFA-3 (3) and FcγRIII (6); reviewed in Refs. 1 and 2).

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The abbreviations used are: PI, phosphatidylinositol; mIg, membrane-bound immunoglobulin; TM, transmembrane; Tyr-Ser and Tyr-Val; Cyto-Δ, cytoplasmic mutant; deleted cytoplasmic domain; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

All transmembrane proteins contain hydrophobic sequences that traverse the lipid bilayer, with charged intracellular and extracellular domains. By comparison, it is thought that prior to PI linkage, immature precursor proteins are processed by deletion of a number of hydrophobic residues at the carboxyl terminus (1, 2, 7). A unique signal sequence for the post-translational modification has not been identified, and it appears likely that a variety of sequences differing in length and primary structure (albeit predominantly hydrophobic) will suffice (1, 7). Of note, a single Asp→Val amino acid substitution converts the PI-anchored Qa-2 antigen to a transmembrane molecule with a three-amino acid (Asn→Arg) cytoplasmic tail (8). It is likely, however, that the mutated Qa-2 residue is distant from the ultimate PI-linkage site, and the putative cytoplasmic and transmembrane domains, including mutated residue, may be deleted prior to attachment of the PI anchor (2). In comparison, a single Ser→Phe substitution in the transmembrane domain of FcγRIII determines membrane topology either by directing PI-linkage processing or by altering the interaction of FcγRIII with the γ-chain of FcεRI (9). In this case, the Ser residue in the immediate extracellular domain may be at or very near the site of PI attachment (9).

Membrane-bound immunoglobulins (mlg) are integral transmembrane proteins on B-cells and are capable of binding and internalizing specific antigen and directing its subsequent processing and presentation to T-cells (10, 11). In addition, cross-linking mlg with specific antigen or anti-Ig antibodies induces a cascade of intracellular signals including an increase in cytoplasmic calcium, altered inositol phosphate metabolism, and expression of oncogene mRNA, resulting in movement of resting B-cells into the cell cycle (reviewed in Ref. 12). All isotypes of mlg have (a) extracellular antigen binding domains; (b) highly conserved, largely hydrophobic transmembrane sequences; and (c) cytoplasmic tails of variable lengths (28–28 residues). IgM and IgD, the antigen receptors on mature, resting B-cells, have conserved three-amino acid (Lys-Val-Lys) cytoplasmic domains (13). Given the short cytoplasmic sequence, it is not surprising that IgM and IgD have no known kinase or other enzymatic activity, and are therefore likely to interact with other transmembrane or cytoplasmic molecules to mediate their known biological effects.

We previously demonstrated that cotransfection of an antigen-specific IgM heavy chain construct (containing wild-type human transmembrane and cytoplasmic domains), with the appropriate antigen-specific k chain into the A20 murine B-cell lymphoma line, faithfully reproduced antigen presentation and signaling functions of mlg (14). Nonconservative mutations affecting the transmembrane Tyr87 (Tyr87→Ser200)
Polyvalent goat anti-mouse IgG2A or anti-human IgM (Southern)
linked immunosorbent assay procedure was adapted from Boom et al.
(16). The rearranged phosphorylcholine-specific heavy chain variable region, derived from the SJ17 plasma celloma, was a gift from Dr. S.-P. Kwan. The sequence, along with the murine heavy chain enhancer and the human heavy chain promoter, was inserted into the pA21 plasmid which contained the human heavy chain exons modified by Bal3I digestion to delete the polyadenylation cleavage site for secreted 

RESULTS AND DISCUSSION

To test the possibility that certain mutant mlg molecules are expressed as PI-anchored proteins, transfected cells were cultured for varying times with and without PI-PLC, and the quantity of released and cell-associated Ig measured by enzyme-linked immunosorbent assay. As shown in Table I, treatment of the Cyto: Δ cells with PI-PLC for 1 h leads to quantitative release of the transfected human IgM. In comparison, the transfected wild-type and TM: YS/VV human

| Table I
| Release of mlg by PI-PLC treatment |
|-----------------|-----------------|-----------------|
| HUMAN IgM | MOUSE IgG2A | HUMAN IgM | MOUSE IgG2A |
| Cell line | Treatment | Superscript | Cell-associated | Mouse IgG2A, superscript |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Wild-type | 1-h PI-PLC | <20 | 240 | 40 |
| | 1-h culture | <20 | 306 | 40 |
| | 24-h culture | <20 | ND* | 40 |
| Cyto: Δ | 1-h PI-PLC | 320 | 40 | <20 |
| | 1-h culture | <20 | 340 | 20 |
| | 24-h culture | 900 | ND | 600 |
| TM: YS/VV | 1-h PI-PLC | <20 | 220 | 20 |
| | 1-h culture | <20 | 340 | 20 |
| | 24-h culture | <20 | ND | 520 |

* ND, not determined.
mlgMs are not released by enzymatic treatment; the endogenous mouse mlgG2A is also unaffected. Interestingly, the Cyto: Δ IgM is also released spontaneously during a 24-h culture, perhaps due to endogenous phospholipase activity. The wild-type and TM: YS/VV transfected clones show no accumulation of human IgM in 24-h supernatants because the transfected human mlgM construct was generated with a deletion of the polyadenylation cleavage site necessary for synthesis of secreted Ig (14). In contrast, the endogenous murine IgG2A is detected in 24-h supernatants from all the cell lines over 24 h, presumably because it can be expressed as a membrane or secreted Ig. These results suggest that the Cyto: Δ human IgM construct is PI-linked, whereas the wild-type and TM: YS/VV clones are insensitive to PI-PLC treatment, and are therefore transmembrane proteins analogous to the endogenous mlgG2A.

To determine the proportion of Cyto: Δ cells with PI-linked mlgM, we measured the PI-PLC-induced loss of mlg by immunofluorescent staining and flow cytometry. Fig. 1 shows a uniform reduction in fluorescent staining for human IgM on the entire Cyto: Δ population after PI-PLC treatment. Wild-type and TM: YS/VV clones show identical staining with or without PI-PLC exposure, confirming that these constructs are PI-PLC-insensitive. Likewise, the endogenous IgG2A is insensitive to PI-PLC on all clones (results not shown). Loss of staining of the PI-linked glycoprotein J11d (24) following PI-PLC hydrolysis confirms that all clones are uniformly capable of PI-linkage, and are comparably sensitive to PI-PLC (results not shown).

By Western blot analysis (Fig. 2A), wild-type (lanes b and f) and TM: YS/VV (lanes d and h) clones have a similar PI-PLC-insensitive doublet corresponding to the human IgM heavy chain, with apparent molecular masses of 74 and 71 kDa. It is not clear whether the doublets represent variably glycosylated molecules or mature cell surface and immature intracellular Ig, although we favor the latter possibility. By comparison, the Cyto: Δ clone (lanes c and g) has a doublet with apparent molecular masses of 72 and 70 kDa, both of which are released after PI-PLC treatment. A band at 72 kDa is present in the supernatant of PI-PLC-treated Cyto: Δ cells only (lane k). Western blots probed for the endogenous murine IgG2A show doublets at 63 and 62 kDa which are insensitive to PI-PLC in all clones (results not shown). The difference of approximately 2 kDa in the apparent molecular mass between the wild-type and Cyto: Δ Ig μ heavy chains is consistent with deletion of most, if not all, of the putative transmembrane 26-amino acid residues prior to PI-linkage.

To verify that the Cyto: Δ IgM is directly PI-anchored, rather than associated indirectly with another PI-linked molecule, we adapted flow cytometric techniques to analyze mlg internalization (Fig. 3). mlg on the clones was cross-linked with either F(ab')2 rabbit anti-mouse γ2A (RAMγ2A, internal control) or F(ab')2 rabbit anti-human IgG (RAHμ). The bound ligand was then allowed to inter-
nalize for increasing periods of time prior to staining for residual surface rabbit Ig using fluoresceinated goat anti-rabbit Ig. For all clones, the endogenous IgG2A is rapidly internalized after cross-linking (represented by a decrease in fluorescence staining intensity; maximum shift occurs by 15 min), in agreement with other published data (10, 11). In control experiments with 1% azide, no internalization is seen after cross-linking (not shown). Wild-type and TM: YS/VV-transfected mIg are also rapidly internalized with kinetics virtually identical to the endogenous IgG2A. In contrast, the PI-anchored Cyto: Δ IgM is slowly internalized, with little diminution of fluorescence intensity at 15 min (Fig. 3) or even at 60 min (not shown). This slow rate of internalization may be largely due to constitutive membrane turnover (25, 26). Comparable results have been obtained with radiiodinated antigens which are bound by the transfected mIgM (results not shown). Of note, while the PI-linked Cyto: Δ mIgM is much less efficient than the transmembrane wild-type mIgM in terms of antigen internalization and subsequent presentation, it is still more efficient than fluid-phase pinocytosis in mock-transfected cells lacking any form of mIgM (14).

Thus, we have shown that tail-deleted IgM mutants, which are defective in intracellular signaling and antigen presentation (14), have been converted from transmembrane to PI-anchored molecules, and are slowly internalized at a rate attributable to normal membrane turnover. These results demonstrate that deletion of the cytoplasmic tail of integral transmembrane proteins may facilitate the processing necessary to generate PI linkage. These results also suggest that PI anchorage may not be dependent on specific signal sequences; in the absence of a charged cytoplasmic tail, the alternative PI linkage may be preferentially used for cell surface interactions. As a practical application, cytoplasmic tail-deleted constructs of a variety of surface molecules (e.g., T-cell antigen receptors) may permit relatively easy generation of soluble forms after PI-PLC treatment.

Furthermore, conversion of mIg from a transmembrane to a PI-linked form interferes with normal signal transduction and efficient antigen internalization, presumably since the mIg can no longer interact with other transmembrane or cytoplasmic proteins important for these functions. Although it has been suggested that PI-linked proteins (specifically Ly-6 and Thy-1) can directly mediate T-cell activation following antibody cross-linking (27, 28), alternative explanations for such “activating” PI-linked molecules include a role in T-cell-accessory cell interactions (29) or surface interaction with other, transmembrane signal-transducing molecules.

Finally, the mIgM mutants Cyto: Δ and TM: YS/VV, both of which are inferior to wild-type constructs in antigen presentation, show distinct cell surface linkages and internalization kinetics. In light of the observation that wild-type and TM: YS/VV clones internalize bound ligand at comparable rates, it is interesting to speculate that the TM: YS/VV mutant may have defective antigen presentation function (14) because of abnormal intracellular targeting after internalization. We are currently investigating such a possibility using subcellular fractionation techniques.

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