Molecular mapping of signals in the Qa-2 antigen required for attachment of the phosphatidylinositol membrane anchor

(phospholipase C/transplantation antigens/exon shuffling/posttranslational modification)

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ABSTRACT   Proteins anchored in the membrane by covalent linkage to phosphatidylinositol (PtdIns) can be released by treatment with purified PtdIns-specific phospholipase C (PtdIns-PLC). A recent survey of leukocyte antigens using flow cytometry has shown that staining of certain Qa antigens was diminished after PtdIns-PLC treatment, but staining of structurally related H-2 antigens was not affected. Therefore, in this study, the sensitivity of cell-surface Qa-2, H-2K^b, and H-2D^b to hydrolysis by PtdIns-PLC was investigated biochemically by immunoprecipitation of radioiodinated molecules from cell lysates or supernatants. Qa-2, but not H-2K^b, was released from the surface of PtdIns-PLC-treated C57BL/10 mouse spleen cells and recovered in the cell supernatants. Similar analysis of thymoma cells transfected with cloned C57BL/10 genes showed that cell-surface Qa-2 molecules encoded by a Q7^b cDNA and the Q7^b or Q9^b gene were sensitive to hydrolysis by PtdIns-PLC, whereas the H-2K^b and H-2D^b gene products were resistant. Using thymoma cells transfected with hybrid genes constructed by exchanging exons between Q7^b and H-2D^b, the signals for PtdIns modification were localized to a defined region of Qa-2. This region differs from H-2D^b most significantly by the presence of a central aspartate residue in the transmembrane segment and in the length of the cytoplasmic portion.

Qa-2 is a cell-surface glycoprotein that is structurally related to the H-2 class I transplantation antigens K, D, and L, in that all contain three homologous external domains (α1, α2, and α3) associated noncovalently with β_2-microglobulin (reviewed in ref. 1). The exon/intron organization of several genes in the Qa region (2–4) is also similar to the H-2K, H-2D, and H-2L genes. Despite these similarities, major differences exist between the expression and function of Qa-2 and H-2K, H-2D, or H-2L (reviewed in ref. 5). Qa-2 shows little polymorphism (two alleles, positive and null) and is expressed only on the surface of defined subpopulations of hematopoietic cells, whereas the K, D, and L antigens are highly polymorphic and are expressed on virtually all adult tissues. Although H-2K, H-2D, and H-2L have been shown to serve well-defined functions in the immune response, the function of Qa-2 has not been ascertained.

Attempts to identify the gene(s) encoding Qa-2 and to study the function and expression of Qa-2 molecules were hindered previously by the inability to express Qa-2 on the surface of L cells transfected with Qa region genes (6–9). By transfection of the thymoma line R1.1, however, cell-surface Qa-2 from the Q7^b gene of C57BL/10 mice has been expressed in a tissue-specific manner (9). We also exchanged gene segments between Q7^b and H-2D^b and mapped the tissue-specific element to a region downstream of a con-

served Stu I site in exon 4, which encodes domain α3. Hybrid genes containing a 3' segment from Q7^b were expressed only on the surface of thymoma cells, whereas those containing an H-2D^b 3' segment (which encodes the transmembrane and cytoplasmic domains) were also expressed on L cells (9).

In our previous study (9), it was unclear if the tissue-specific expression of cell-surface Qa-2 involved posttranscriptional or postranslational events. RNA blot-hybridization analysis of thymoma and L cells transfected with the Q7^b gene showed multiple RNA species, suggesting alternate mRNA processing in both cell lines. Although both lines showed similar levels and species of mRNA, L-cell transfectants expressed no detectable levels of cell-surface Qa-2 antigen. In contrast, L cells transfected with a Q7^b cDNA isolated from a cytotoxic T lymphocyte library expressed low but significant levels of cell-surface Qa-2, suggesting that the tissue-specific expression of cell-surface Qa-2 may be explained partly at the mRNA level. However, thymoma cells transfected with this same Q7^b cDNA construct expressed relatively high levels of Qa-2 on their surface, suggesting that thymoma cells but not L cells could provide an additional posttranslational requirement.

Until recently, it was believed that all integral plasma membrane proteins were anchored by way of hydrophobic amino acid (transmembrane) segments interacting noncovalently with the hydrophobic tails of membrane lipids. In the past several years, however, a number of proteins have been shown to be anchored solely by covalent linkage to a glycophospholipid moiety containing phosphatidylinositol (PtdIns) (reviewed in refs. 10 and 11). Although the chemical nature of this moiety is known for only a few proteins, evidence for linkage to PtdIns has come from studies demonstrating the removal of proteins from the membrane by purified PtdIns-specific phospholipase C (PtdIns-PLC). Thus, this enzyme has proven to be a valuable tool for assaying molecules linked to PtdIns.

Recently, Stiernberg et al. (12) showed by flow cytometry that fluorescent staining of Qa-2 on spleen cells was diminished after treatment with PtdIns-PLC and, therefore, at least some Qa-2 molecules may be linked to PtdIns. Biochemical studies (reviewed in ref. 13) have shown that transplantation antigens, such as H-2K, H-2D, and H-2L, are anchored in the plasma membrane by way of a typical hydrophobic membrane-spanning region of the polypeptide and are resistant to PtdIns-PLC treatment (12). H-2K, H-2D, and H-2L also have cytoplasmic segments of 20–35 amino acids, but deletion mutagenesis of most of this segment in H-2L^d failed to prevent its expression on the surface of L cells (14). The apparent linkage of Qa-2 to PtdIns is intrigu-

Abbreviations: mAb, monoclonal antibody; PtdIns, phosphatidylinositol; PtdIns-PLC, PtdIns-specific phospholipase C.
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ing because the $Q7^b$ gene (4) and $Q7^b$ cDNA (9) can encode a putative transmembrane segment and a cytoplasmic peptide three amino acids long. However, studies of Thy-1 and the variant surface glycoprotein of trypanosomes, for which genomic, cDNA, and protein sequences are available, have shown that a carboxyl-terminal segment of the protein is removed before addition of the PtdIns anchor (reviewed in refs. 10 and 11). Comparison of the carboxyl-terminal regions predicted from genomic and/or cDNA sequences of these and other proteins anchored by PtdIns (10) failed to reveal consensus amino acids signaling PtdIns modification but showed that all have truncated transmembrane segments.

In order to determine the fate of class I antigens after PtdIns-PLC treatment, we immunoprecipitated ^{125}I-labeled surface molecules from lysates of intact cells or from cell supernatants. Using this method, we investigated the PtdIns-PLC sensitivity of Qa-2, H-2K^b, and H-2D^b antigens expressed on spleen cells or thymoma cells transfected with cloned class I genes. By analyzing transfectants expressing hybrid genes between $Q7^b$ and H-$2D^b$, this approach has allowed us to localize the signals in Qa-2 required for its modification by PtdIns.

MATERIALS AND METHODS

Cell Lines. The Qa-2$^-$, H-2^k murine thymoma line R1.1 (ATCC TIB 42) was transfected with various H-2^b class I gene constructs by electroporation, and stable transfectants were selected in G418 sulfate (GIBCO) at 1 mg/ml as described (9). As described previously (9), the expression vector Q7^{b}cDNAMo was constructed by inserting a full-length $Q7^b$ cDNA plus 3' splice and polyadenylylation signals from simian virus 40 into pTCFMo, a vector containing the Moloney virus enhancer and promoter. D^{b}Mo and Q7^{b}GeneMo consist of the H-$2D^b$ and $Q7^b$ genomic genes in the vector pTCFMo. Q7^b/D^{b}Mo and D^b/Q7^{b}Mo are hybrid genes constructed by exchanging exons encoding domains $\alpha1$, $\alpha2$, and the amino-terminal half of $\alpha3$ from one parent with the remainder from the other parent (9). For this study, R1.1 cells were transfected with Q9^{b}GeneMo, a construct similar to Q7^{b}GeneMo. R1.1 cells transfected with H-$2K^b$ were a generous gift of Hamish Allen (Biogen Research). C57BL/10 spleen cells were used as freshly prepared suspensions.

Radioiodination. Cell-surface proteins were labeled with ^{125}I (Dupont/New England Nuclear) using lactoperoxidase as described previously (15).

Immunoprecipitation and NaDodSO₄/PAGE. H-2K^b and Qa-2 as well as Q7^b/D^b hybrid proteins were immunoprecipitated from cell supernatants or Nonidet P-40 cell extracts using the monoclonal antibody (mAb) 20-8-4 (16, 17), a gift of David Sachs (Immunology Branch, National Cancer Institute). H-2D^b and D^b/Q7^b hybrid proteins were immunoprecipitated using mAb B22.249 (17). Antibody–antigen complexes were collected on protein A-Sepharose (Pharmacia) and were analyzed by NaDodSO₄/PAGE on 10% gels as described previously (19). Dried gels were autoradiographed for 5–10 days at −70°C using Kodak XAR film and a Dupont Cronex intensifying screen.

PtdIns-PLC Treatment. PtdIns-PLC was purified from culture supernatants of *Bacillus thuringiensis* according to a modified procedure (M.G.L., unpublished results) used previously for purification of *Staphylococcus aureus* PtdIns-PLC (20). PtdIns-PLC activity was analyzed in the presence of 0.1% deoxycholate at pH 7 using [^{3}H]phosphatidylinositol as substrate. One unit of PtdIns-PLC activity is defined as the amount required to hydrolyze 1 μmol of PtdIns per min.

PtdIns-PLC treatment was performed by culturing ^{125}I-labeled cells (2 × 10^6) at 37°C in 0.5 ml of RPMI 1640 growth medium containing 10% fetal bovine serum and PtdIns-PLC as indicated. Mock treatment was performed by culturing the cells in growth medium without PtdIns-PLC. After treatment, the cells were centrifuged, and the supernatants were collected. The pellet was washed twice and then was lysed in 0.5 ml of extraction buffer as described (15).

RESULTS

Qa-2 but Not H-2K^b on Spleen Cells Is Sensitive to PtdIns-PLC. Previous analysis (12) by flow cytometry has shown that the staining of Qa-2 antigen was diminished after treatment of spleen cells with PtdIns-PLC, suggesting that some Qa-2 molecules may be anchored by linkage to PtdIns. Based on other PtdIns-anchored proteins that have been characterized, PtdIns-PLC treatment should release Qa-2 into the supernatant. The PtdIns-PLC preparation contains little, if any, protease; the released proteins are therefore unlikely to be degraded and should be approximately the same molecular mass as the membrane-bound form. Proteins not anchored by PtdIns, such as H-2K^b and H-2D^b, should remain intact on the cell surface after treatment.

In order to test these predictions, we investigated whether intact Qa-2 and H-2K^b molecules are released by PtdIns-PLC from the cell surface of ^{125}I-labeled C57BL/10 spleen cells. The association of Qa-2 and K^b with either the supernatant or the pellet fractions from mock-treated or PtdIns-PLC-treated cells was determined by NaDodSO₄/PAGE analysis of immunoprecipitates using the mAb 20-8-4. This mAb reacts with both the ≈40-kDa Qa-2 and the ≈45-kDa K^b glycoproteins and, thus, can be used to analyze the sensitivity of each molecule from the same population of cells.

The ≈40-kDa Qa-2 glycoprotein is associated with the cell pellet in the mock-treated group, but it is found in the supernatant of the PtdIns-PLC-treated group (Fig. 1). This result suggests that most of the Qa-2 antigen on spleen cells is anchored in the membrane by way of linkage to PtdIns. In contrast, the ≈45-kDa K^b glycoprotein is associated only with the cell pellet in both groups. This result is expected since K^b is known to be anchored by way of a hydrophobic transmembrane segment (21).

Qa-2 Expressed from a Q7^b cDNA Is Sensitive to PtdIns-PLC. We reported previously that R1.1 thymoma cells transfected with a Q7^b cDNA construct (clone Q7c26R) expressed relatively high levels of cell-surface Qa-2 antigen, as detected by binding of specific mAbs (9). This has been confirmed subsequently by immunoprecipitation and NaDod-
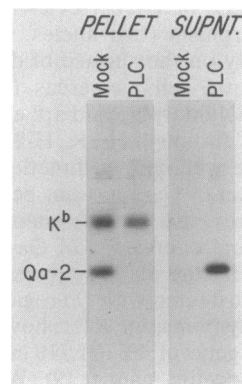

FIG. 1. PtdIns-PLC sensitivity of Qa-2 on the surface of spleen cells. Radioiodinated spleen cells from C57BL/10 mice were cultured for 1 hr in the absence of PtdIns-PLC (Mock) or in the presence of 0.2 unit of PLC (PLC). Culture supernatants (Supnt.) and cell lysates (Pellet) were immunoprecipitated with 0.1 μl of 20-8-4 ascites fluid and analyzed by NaDodSO₄/PAGE on 10% gels.

Immunology: Waneck et al.

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SO$_4$/PAGE of ^{125}I-labeled surface proteins (22). In order to determine if Qa-2 molecules expressed by Q7c26R cells were sensitive to PtdIns-PLC as were those expressed on spleen cells, we radioiodinated cell-surface proteins of this clone and analyzed the effect of various levels of PtdIns-PLC and various incubation periods (data not shown). As little as 0.008 unit of PtdIns-PLC released nearly all of the cell-surface Qa-2 into the supernatant after 60 min. Maximal release was obtained with 0.04 unit, but even at the highest dosage (1.0 unit) a very small amount of Qa-2 still appeared to remain on the surface.

To be certain that the incubation period was sufficient, we determined the kinetics of release by incubating Q7c26R cells at various times in the presence or absence of 0.2 unit of PtdIns-PLC (data not shown). Cells that were mock treated showed no significant release of Qa-2 into the supernatant even after 4 hr of culture, and at 60 min the amount of Qa-2 associated with the cell pellet was the same as that at the zero time point. In contrast, PtdIns-PLC-treated cells placed on ice immediately after addition of the enzyme already showed significant release. Maximal release was obtained after only 5 min and did not increase noticeably

even after 4 hr of incubation. Thus, the release of Qa-2 by PtdIns-PLC is very rapid, and incubation of the cells for 60 min in the presence of 0.04 unit of enzyme is more than sufficient to obtain maximal release.

PtdIns-PLC Sensitivity of Molecules Encoded by Hybrid Genes. In order to investigate which region of Qa-2 contains the signals required for its posttranslational modification by PtdIns, we tested a panel of R1.1 clones transfected with genes that express either native class I molecules or hybrid molecules between $Q7^b$ and H-$2D^b$. Assuming that no unusual splicing events occur in transcripts from these genes and based on the length of the open reading frames predicted from the cDNAs, mature polypeptides encoded by H-$2D^b$ and $Q7^b$ should have the amino acid sequence shown in Fig. 2. The hybrid polypeptides that result from exchange of sequences using a conserved Stu I site in the exon encoding domain α3 can be deduced from Fig. 2. Overall, the positional identity of the two proteins is about 80%. The relative levels and sizes of the polypeptides expressed on the surface of each transfectant are shown in Fig. 3.

Using mAbs specific for determinants in the α1/α2 domains of K^b and Qa-2 (mAb 20-8-4) or D^b (mAb 22.249), we


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        ┌ Domain α1 →
D^b   1 GPHSMRYFETAVSRPGLEEPRYISVGYVDNKEFVRFDSDAENPRYEPRAP 50
        | || || |||||||| || ||||||||    |||||||||||| ||||
Q7^b  1 GQHSLQYFHTAVSRPGLGEPWFISVGYVDDTQFVRFDSDAENPRMEPRAR 50

                                    ┌ Domain α2 →
     51 WMEQEGPEYWERETQKAKGQEQWFRVSLRNLLGYYNQSAGGSHTLQQMSG 100
        |||||||||||||| ||| || || |||  ||||| ||||||| | |
     51 WMEQEGPEYWERETQIAKGHEQSFRGSLRTAQSYYNQSKGGSHTLQWMYG 100

    101 CDLGSDWRLLRGYLQFAYEGRDYIALNEDLKTWTAADMAAQITRRKWEXS 150
        || |||| ||||||||||||||||||||||||| ||| ||||||||||||
    101 CDMGSDGRLLRGYLQFAYEGRDYIALNEDLKTWTAVDMAAQITRRKWEQA 150

                            ┌ Domain α3 →
    151 GAAEHYKAYLEGECVEWLHRYLKNGNATLLRTDSPKAHVTHHPRSKGEVT 200
        | || |||||  |  | ||| | |||||| |||||||||||| | ||
    151 GIAEKDQAYLEGTCMQSLRRYLQLGKETLLRTDPPKAHVTHHPRSYGAVT 200
                            ↑

                    Stu I
                    ↓
    201 LRCWALGFYPADITLTWQLNGEELTQDMELVETRPAGDGTFQKWASVVVP 250
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    201 LRCWALGFYPADITLTWQLNGEELTQDMELVETRPAGDGTFQKWASVVVP 250

                                ┌ Transmembrane →
    251 LGKEQNYTCRVYHEGLPEPLTL...RWEPPPSTDSYMVIVAVLGVLGAMA 297
        ||||||||| | ||||||||||   |||||| | | | || ||| |
    251 LGKEQNYTCHVNHEGLPEPLTLRWGRWEPPPYTVSNMATIAVVVDLGAVA 300

        ┌ Cytoplasmic →
    298 IIGAVVAFVMKRRRNTGGKGGDYALAPGSQSSEMSLRDCKA 338
        ||||||||||| ||
    301 IIGAVVAFVMNRR............................. 313
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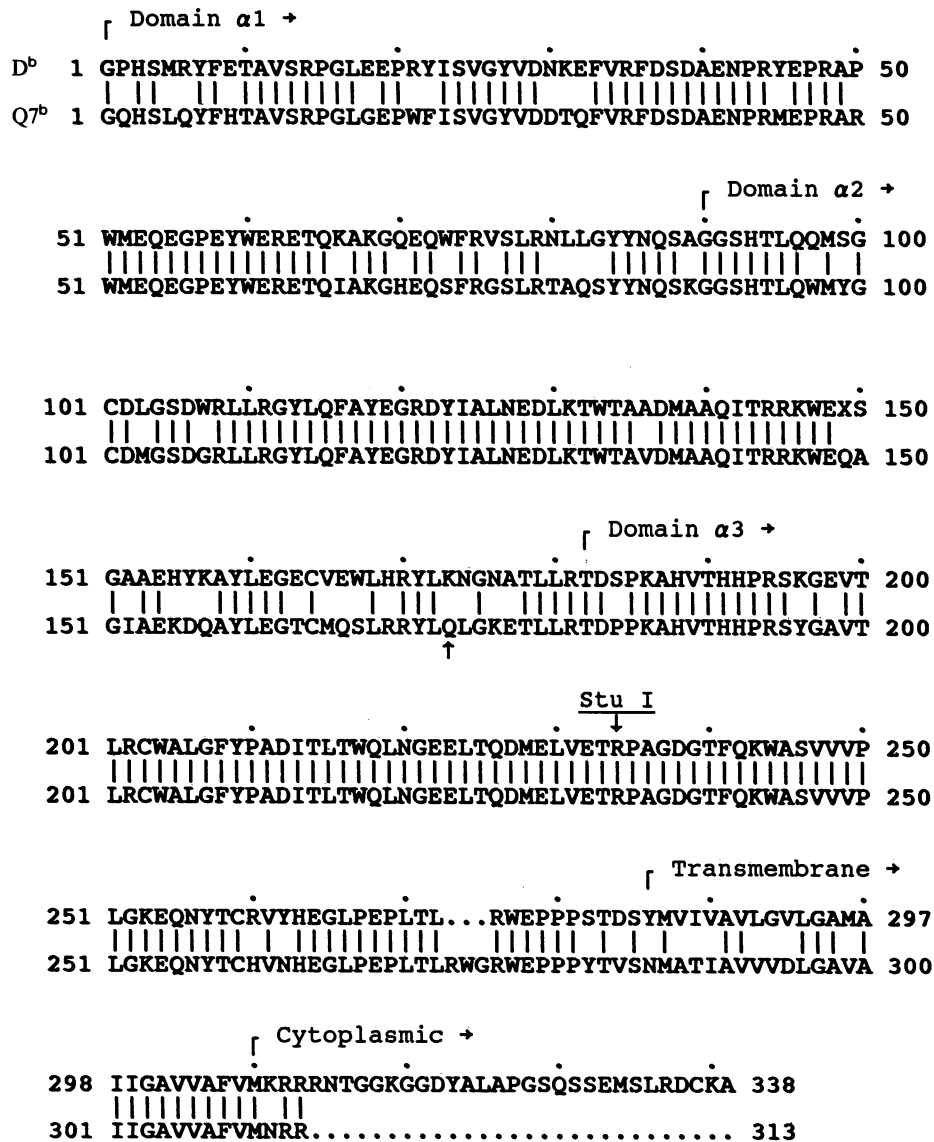
Fig. 2. Comparison of H-2D^b (upper sequence) and Q7^b (lower sequence) polypeptides. The mature protein sequences are predicted from the cDNA sequences of H-2D^b (ref. 23 and G.L.W., unpublished results) and Q7^b (9). The domain structures and the location of the Stu I site used for exon exchange are shown. Arrow indicates glutamine at position 173 in Q7^b, which is glutamate in Q9^b (4).
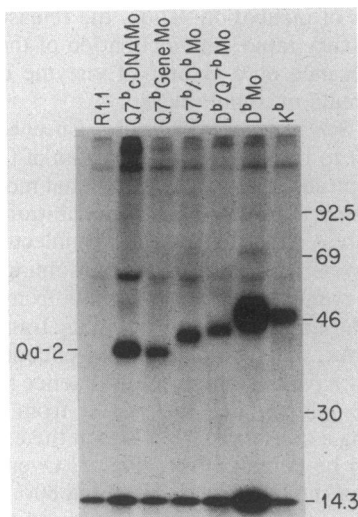
FIG. 3. Relative sizes and levels of cell-surface glycoproteins encoded by transfected class I genes. R1.1 cells transfected with C57BL/10 class I constructs were radioiodinated, lysed, immunoprecipitated with 0.1 μl of 20-8-4 ascites fluid or 50 μl of B22.249 culture supernatant, and analyzed by NaDodSO₄/PAGE. Molecular mass markers (in kDa) are indicated at right.

immunoprecipitated the supernatants and pellets of ¹²⁵I-labeled transfectants that were either mock treated or incubated for 2 hr with 0.2 unit of PtdIns-PLC and analyzed these samples by NaDodSO₄/PAGE. Qa-2 molecules encoded by Q7ᵇcDNAMo and Q7ᵇGeneMo are released into the supernatant after treatment with PtdIns-PLC as shown in Fig. 4. Significantly, neither the Kᵇ nor Dᵇ molecules appear to be sensitive to this enzyme, and they remain associated mostly with the cell pellet. It is important to note that the Dᵇ/Q7ᵇ hybrid containing the carboxyl terminal portion of Q7ᵇ is sensitive to PtdIns-PLC, but the reciprocal construct containing the carboxyl-terminal portion of Dᵇ is resistant, as is the Dᵇ parent molecule. This experiment therefore shows that the signals for modification by PtdIns must be contained in the region downstream of the hybrid site (see Fig. 2),

which represents only a portion of the α3 domain and the transmembrane/cytoplasmic segment.

The *Q9ᵇ* Gene also Encodes PtdIns-PLC-Sensitive Qa-2. Peptide mapping experiments by Sherman *et al.* (22) have shown that either *Q7ᵇ* and/or *Q9ᵇ* encode Qa-2 on spleen cells. The restriction enzyme map of *Q9ᵇ* is more similar to that of *Q7ᵇ* than other *Qa* region genes (24). The complete sequence of *Q7ᵇ* is known (4); however, only the sequence of exons 1, 2, 3, 6, and 8 of *Q9ᵇ* have been determined (4, 25). In these coding regions, there is only a single nucleotide difference between *Q7ᵇ* and *Q9ᵇ* in exon 3 that results in a substitution of glutamine in Q7ᵇ glutamic acid in Q9ᵇ (see Fig. 2). From analysis of the *Q7ᵇ* hybrid gene products above, it is the α3 domain and/or transmembrane/cytoplasmic segment of Qa-2 that appears to contain the signals for PtdIns modification. These regions are encoded by exons 4 and 5, which have not been sequenced in *Q9ᵇ*.

Since a population of Qa-2 antigens on the surface of spleen cells appears to be PtdIns-PLC-resistant (12), it was therefore of interest to characterize the PtdIns-PLC sensitivity and molecular mass of the *Q9ᵇ* gene product. The effect of PtdIns-PLC treatment on cell-surface Qa-2 expressed by R1.1 cells transfected with similar *Q7ᵇ* or *Q9ᵇ* constructs are shown in Fig. 5. Qa-2 encoded by *Q9ᵇ* comigrates with that of *Q7ᵇ* and also is PtdIns-PLC sensitive (Fig. 5). Thus, it appears that both of these genes have the potential to encode PtdIns-anchored Qa-2 molecules on the surface of spleen cells.

DISCUSSION

Using immunochemical methods, we have confirmed that the majority of Qa-2 on the surface of spleen cells is anchored by linkage to Ptd-Ins. In addition, we have shown that thymoma cells transfected with cloned Q7ᵇ cDNA, the *Q7ᵇ* gene, or the *Q9ᵇ* gene express cell-surface Qa-2 molecules that are sensitive to release by PtdIns-PLC. In contrast, the majority of Kᵇ molecules on spleen cells or thymoma cells transfected with a cloned *H-2Kᵇ* gene and the majority of Dᵇ molecules expressed on transfected cells are resistant to release by PtdIns-PLC.
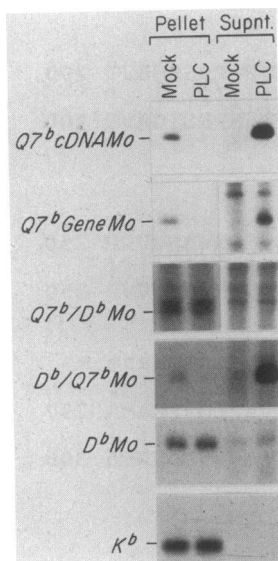

FIG. 4. PtdIns-PLC sensitivity of cell-surface glycoproteins encoded by transfected class I genes. Radioiodinated R1.1 transfectants were cultured for 2 hr in the absence of PtdIns-PLC (Mock) or in the presence of 0.2 unit of PLC (PLC). Culture supernatants (Supnt.) and cell lysates (Pellet) were immunoprecipitated and electrophoresed as described in Fig. 3.
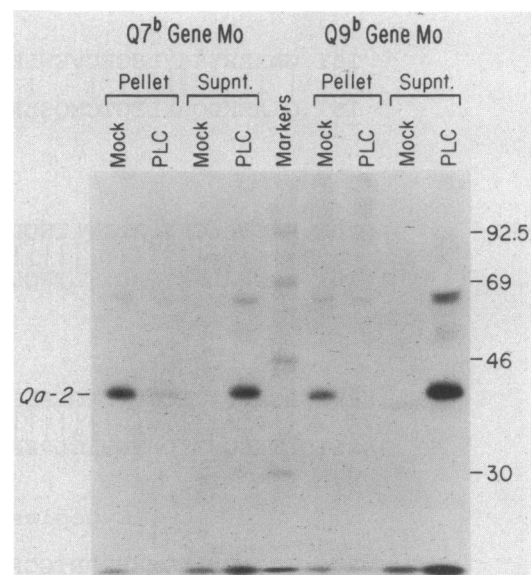

FIG. 5. PtdIns-PLC sensitivity of cell-surface Qa-2 molecules encoded by homologous *Qa* region genes. Radioiodinated R1.1 cells transfected with Q7ᵇGeneMo or Q9ᵇGeneMo were analyzed as described in Fig. 4. Molecular mass markers are in the center lane and are indicated at right in kDa.

In general, the significance of PtdIns modification is obscure, although in some cases PtdIns-anchored proteins are associated with cellular activation events (26–28). In this respect, it is interesting that Qa-2 is "secreted" into the media of concanavalin A-activated spleen cells (29). Biosynthetic studies of its release suggest that Qa-2 is first expressed on the cell surface and then processed to a soluble form of slightly lower molecular mass (30). Since various forms of signal transduction and metabolism of inositol-containing lipids are intimately connected (31), it is tempting to speculate that activation of extracellular anchor-degrading phospholipases may account for the release of Qa-2. Phospholipases C and D with specificity for PtdIns anchors have been identified in liver plasma membranes (32) and in mammalian serum (38). Perhaps diacylglycerol or phosphatidic acid liberated into the membrane by these enzymes may influence intracellular pathways by activating protein kinase C or inositol phospholipid metabolism (33–35). Alternatively, the soluble form of Qa-2 may have a function that remains to be defined.

The alignment of carboxyl-terminal sequences predicted for precursors of various PtdIns-anchored proteins has failed to reveal consensus amino acids that might be signals for this modification (10). Because the $Q7^b$ and D^b amino acid sequence differences are known (4, 9, 23), the hybrid proteins have defined a specific region containing these potential signals (see Fig. 2). Within this region, the two proteins differ most significantly by the presence of aspartate at position 295 in $Q7^b$, which is valine in D^b and other class I polypeptides (reviewed in ref. 36), and in the length of their cytoplasmic segments. Most of the other differences appear to represent conservative substitutions or normal diversity seen among proteins, such as H-2K, H-2D, and H-2L (36), and may not be significant in signaling PtdIns modification of Qa-2.

Although the precise location and nature of these signals remain obscure, the Qa/H-2 system—in which markedly different molecular mechanisms are utilized for anchoring similar extracellular domains—appears well suited for investigating PtdIns modification by further exon shuffling or site-directed mutagenesis. In addition, the ability of Qa-2 or the hybrid proteins to be modified by PtdIns correlates well with their ability to be expressed on thymoma cells but not on fibroblasts (9). These observations suggest an explanation for the tissue-specific expression of cell-surface Qa-2 *in vivo* and may provide a clue for determining its function.

Note. While this paper was under review, Stroynowski *et al.* (37) published a paper reporting similar results.

Note Added in Proof. Davitz *et al.* (39) also have identified a PtdIns-specific phospholipase D in human serum.

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