

# The Glycosyl Phosphatidylinositol Anchor Is Critical for Ly-6A/E-mediated T Cell Activation

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**Abstract.** Ly-6E, a glycosyl phosphatidylinositol (GPI)-anchored murine alloantigen that can activate T cells upon antibody cross-linking, has been converted into an integral membrane protein by gene fusion. This fusion product, designated Ly-6ED<sup>b</sup>, was characterized in transiently transfected COS cells and demonstrated to be an integral cell surface membrane protein. Furthermore, the fusion antigen can be expressed on the surface of the BW5147 class "E" mutant cell line, which only expresses integral membrane proteins but not GPI-anchored proteins. The capability of this

fusion antigen to activate T cells was examined by gene transfer studies in D10G4.1, a type 2 T cell helper clone. When transfected into D10 cells, the GPI-anchored Ly-6E antigen, as well as the endogenous GPI-anchored Ly-6A antigen, can initiate T cell activation upon antibody cross-linking. In contrast, the transmembrane anchored Ly-6ED<sup>b</sup> antigen was unable to mediate T cell activation. Our results demonstrate that the GPI-anchor is critical to Ly-6A/E-mediated T cell activation.

**T**HE murine Ly-6A/E antigen is a member of the Ly-6 multigene family which consists of approximately 20 genes located within a region of ~500 kb on chromosome 15 (14, 15; Philbrick, W. M., and A. L. M. Bothwell, unpublished results). The known proteins encoded in this locus are associated with cells of the hematopoietic lineage and their expression is developmentally regulated in a cell type-specific manner (11, 23, 28). Recently, it has been shown that Ly-6A/E belongs to a class of surface antigens which are anchored to the plasma membrane through a glycosylphosphatidylinositol (GPI)<sup>1</sup> moiety (23). This GPI tail is rapidly added to the nascent protein through a posttranslational modification (17, 18, 25).

Physiological triggering of T cells is mediated through an antigen-specific T cell receptor/CD3 complex. However, cross-linking of the Ly-6A/E antigen on the cell surface has also been shown to activate T cells, resulting in its designation as "T cell activation protein" (TAP) (21, 23). Two other unrelated T cell surface antigens, Thy-1 and Qa-2, are also anchored to the cell surface by a GPI tail, and like Ly-6, can activate T cells via antibody cross-linking (12, 24). This recurrent relationship between triggering capability and attachment structure has led to the suggestion that the GPI anchor may be involved in the signal transduction (4, 22, 23).

To determine whether the GPI anchor of the Ly-6A/E molecule is essential for its role in T cell activation, we have undertaken gene transfer studies in D10, an antigen-specific

type 2 T cell helper clone. This clone expresses high levels of surface Ly-6A antigen, which can be distinguished from its allelic form, Ly-6E by appropriate mAbs (see Results). D10 offers the advantages of a cloned cell line, and yet resembles isolated T cells more closely than either tumor lines or T cell hybridomas with regard to the physiological conditions for activation. In addition, the immunologic properties of the D10 cells have been well characterized (9). Thus, D10 transformants, which express mutagenized Ly-6E molecules, will provide an ideal system in which to investigate the functions of the Ly-6A/E alloantigen.

We have previously used site-directed mutagenesis to elucidate the nature of the signal required for GPI modification of the Ly-6E antigen (25). These studies indicated that mutations of the carboxy terminus of the Ly-6E molecule can partially inhibit or even abolish GPI modification, and further revealed the presence of two signals which are required for proper cleavage and addition of the GPI tail. To investigate the functional significance of the different membrane anchors (integral membrane versus GPI anchor) of the Ly-6E molecule, we have successfully constructed and expressed a transmembrane form of the Ly-6E antigen. The T cell activation capability of this transmembrane Ly-6E antigen and GPI-anchored Ly-6A and E antigens were examined in an antigen-specific T helper clone.

## Materials and Methods

### Cell Culture

COS cells and BW5147 class "E" mutant cells (provided by Dr. R. Hyman,

1. *Abbreviations used in this paper:* GPI, glycosyl phosphatidylinositol; TAP, T cell activation protein.

Salk Institute, San Diego) are maintained in DME plus 10% FCS. The D10 G4.1 T cell clone was generously provided by Dr. C. Janeway (Yale University, New Haven) and is maintained in Click's EHAA medium containing 10% FCS and 5% polyclone (Collaborative Research, Lexington, MA) as described (9). This clone is regularly stimulated by antigen (conalbumin; Sigma Chemical Co., St. Louis, MO) and spleen cells from an H-2<sup>k</sup> mouse every 3–4 wk.

### Construction of the Ly-6E<sup>D<sup>b</sup></sup> Fusion Plasmid

To make a fusion antigen of Ly-6E and MHC class I D<sup>b</sup>, a unique Acc I restriction site was introduced into the Ly-6E cDNA as well as into the MHC class I D<sup>b</sup> gene by site directed mutagenesis as previously described (13, 25). The oligonucleotide used for the mutagenesis was: 5'-AAGCAC-CCCTGCCATGGTATACGGGATCCCTCCATGGGAACCTGTCGATTGCAGAGATCTTCTGGCAACA-3' for Ly-6E and 5'-TGGGAGCTCCTCCGTATACTGACTCTTAC-3' for the D<sup>b</sup> gene. As shown in Fig. 1 A, the Eco RI-Acc I fragment purified from the Ly-6E-Acc I plasmid and the Acc I-Eco RI fragment of plasmid pBG-D<sup>b</sup>-Acc I were ligated into the transient expression vector JC119R at the unique Eco RI site (25). The pcDL-SR $\alpha$ -Ly-6E<sup>D<sup>b</sup></sup> plasmid was constructed by subcloning the Ly-6E<sup>D<sup>b</sup></sup> fusion gene into the vector pcDL-SR $\alpha$  at the unique Eco RI site (27).

### DNA Transfection

Transfection of COS 1 cells was performed as described (25). For transfection of the BW5147 "E" mutant cell line, cells (10<sup>7</sup>) were washed with PBS and resuspended in 0.4 ml 2 $\times$  HBSS (137 mM NaCl, 5 mM KCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM dextrose, 20 mM Hepes pH 7.05). Plasmid (30  $\mu$ g) pcDL-SR $\alpha$ -Ly-6E<sup>D<sup>b</sup></sup> and 3  $\mu$ g pSV2neo were precipitated with ethanol and resuspended in 0.4 ml H<sub>2</sub>O. Cells and DNA were mixed in an 0.4-cm cuvette and incubated on ice for 10 min before electroporation. An 11.2-ms pulse was delivered at 325 V, 960  $\mu$ F using an electroporator (Bio-Rad Laboratories, Richmond, CA). The cells were returned to ice for 10 min, then plated into 96-well plates at 3  $\times$  10<sup>4</sup> cells per well in DME/5% FCS. 2 d later, 1 mg/ml G418 was added to select transfectants. For the D10 T cell transfection, cells were washed once with PBS containing 10 mM MgCl<sub>2</sub> and resuspended in above buffer at 1.5  $\times$  10<sup>7</sup> cell per ml. Cells (0.7 ml) were mixed with 30  $\mu$ g plasmid DNA and 3  $\mu$ g pSV2neo DNA in an 0.4-cm cuvette at room temperature for  $\sim$ 9 min and on ice for 1 min. An 0.5-ms pulse was delivered at 1.24 kV, and 25  $\mu$ F using an electroporator (Bio-Rad). The cells were placed on ice for 10 min, then incubated at room temperature for 10 min and finally diluted 1:5 in the Click's medium for another 25 min. The cells were plated in 96-well plates in Click's medium containing 10% FCS, 5% Polyclone, and 2.5  $\mu$ g per ml Con A (Sigma Chemical Co.). After 72 h, the transformants were selected with medium containing 0.4 mg/ml G-418 (Gibco Laboratories, Grand Island, NY).

### Treatment of Cells with Phosphatidylinositol-specific Phospholipase C (PI-PLC)

Purified PI-PLC was a gift from Dr. Martin Low (Columbia University College of Physicians and Surgeons). Cells ( $\sim$ 2.6  $\times$  10<sup>5</sup> in a 3.5-cm dish) were labeled for 2 h with [<sup>35</sup>S]cysteine and then washed twice in PBS. Finally, 0.4 ml DMEM/5% FCS containing 2  $\mu$ l PI-PLC was added. After 2 h at 37°C, the medium was collected for immunoprecipitation. Controls were processed as above, but without the addition of enzyme.

### Microsome Isolation and Proteolysis with Proteinase K

Semi-confluent COS cells on 6-cm dishes were transfected with 20  $\mu$ g JC119R-Ly-6E<sup>D<sup>b</sup></sup> DNA. After 40 h, cells labeled with [<sup>35</sup>S]cysteine were scraped off the plate and resuspended in 1.2 ml of 15 mM NaCl. The cells were broken by 20 strokes with a Dounce homogenizer. Samples were aliquoted into three tubes, one with 20  $\mu$ g/ml proteinase K, one with 20  $\mu$ g/ml proteinase K plus NP-40, and one without addition. These aliquots were incubated at 0°C for 90 min. The reaction was stopped by adding 0.1 mM PMSF. The samples were then sedimented through 10% sucrose in saline solution in a rotor (model SW55; Beckman Instruments, Fullerton, CA) at 45 krpm for 4 h at 4°C. The pellets were dissolved in lysis buffer and centrifuged in a microfuge for 5 min to remove nuclei and debris. The supernatants were immunoprecipitated with SK70.94 mAb and subjected to gel electrophoresis as described previously (25). In this procedure, any membrane associated Ly-6E<sup>D<sup>b</sup></sup> molecules produced after NP-40 treatment that were not precipitated during sucrose gradient centrifugation were not analyzed.

### Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was performed as described (6). The transfected cells were fixed with 3% paraformaldehyde for 20 min at room temperature or overnight at 4°C. For surface staining, the fixed cells were washed with staining buffer (1 $\times$  PBS plus 10 mM glycine) for 10 min and incubated with a 1:400 dilution of SK70.94 (anti-Ly-6E mAb) for 10 min in staining buffer. For internal staining, the cells were permeabilized with 0.1% Triton X-100 in staining buffer for 5 min before incubating with the antibody. The free antibodies were washed away by rinsing with staining buffer. The intact or permeabilized cells were further incubated with a 1:50 dilution of FITC-conjugated rabbit anti-mouse IgG antibody for 10 min. After removing the excess secondary antibody, the cells were visualized under UV light in a fluorescence microscope.

### Proliferation Assay

D10 cells were incubated with mAbs for 45 min and the free antibodies were removed by washing with the medium. The antibody coated D10 cells were then cultured in triplicate in 96-well round-bottomed plates in Click's EHAA medium plus 10% FCS and 6 pg/ml recombinant IL 1 (Sigma Chemical Co.) in the presence or absence of 10  $\mu$ g/ml rat anti-mouse immunoglobulin F(ab)<sub>2</sub> as cross-linker (Accurate Chemical and Scientific Corp., Westbury, NY). The cells were cultured at 37°C for 48 h and then pulsed with [<sup>3</sup>H]TdR for an additional 6 h and harvested by using an LKB wallac 1295-001 cell harvester (LKB Instruments, Gaithersburg, MD). The radioactivity incorporation into DNA was measured by scintillation counting. The D10 transfectants were expanded and grown for 2 wk in the absence of G-418 before performing activation studies to avoid possible drug effects.

### Bioassay of Lymphokine Secretion

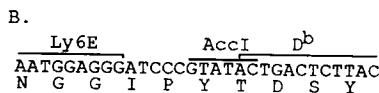
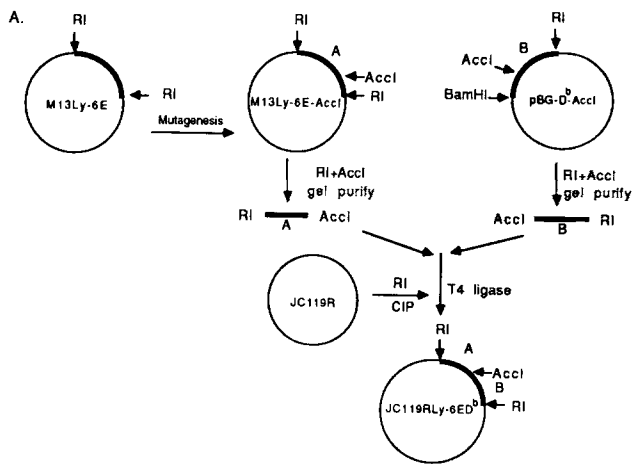
The presence of IL-4 was determined by measuring the proliferation of an IL 4-dependent cell line, CTLL-20 (19). Briefly, D10 cells or its derived transfectants were stimulated by mAbs as described above. After 24 h, the supernatants were harvested and tested for lymphokine activity. CTLL-20 cells (10<sup>4</sup>/well) were incubated for 42 h in a 1:4 dilution of mAb-stimulated D10 supernatant. The cells were pulsed with <sup>3</sup>H-TdR for an additional 6 h and the <sup>3</sup>H incorporation into CTLL cells was measured.

## Results

### Expression of the Ly-6E<sup>D<sup>b</sup></sup> in COS Cells

The construction of the Ly-6E<sup>D<sup>b</sup></sup> chimeric molecule and the junction between Ly-6E and the MHC class I D<sup>b</sup> gene are illustrated in Fig. 1. 20 amino acids were deleted from the carboxy-terminal region of the wild-type Ly-6E antigen, which was then linked to the transmembrane and cytoplasmic regions of the D<sup>b</sup> gene by three amino acids, "IPY," from the restriction linker sequence (Fig. 1 B). The fusion gene retains the putative GPI processing site from Ly-6E, which is then followed by a long stretch of hydrophobic residues as in the wild-type Ly-6E sequence. This fusion gene differs from a typical GPI-anchored protein, however, in that it contains a charged cytoplasmic domain at the carboxy terminus.

The expression of this fusion antigen in transfected COS cells was assayed by metabolic labeling with [<sup>35</sup>S]cysteine and subsequent immunoprecipitation using our previously established conditions (25). As shown in Fig. 2 A, a single protein was detected by immunoprecipitation from the Ly-6E<sup>D<sup>b</sup></sup>-transfected COS cells, and it migrates with an apparent molecular mass of  $\sim$ 18 kD on SDS-PAGE, as compared with 14–15 kD for the wild-type Ly-6E antigen. Since the single precipitated species appears larger than the wild-type antigen, it is likely that the normal GPI processing site in Ly-6E is not utilized. This antigen does not appear to be secreted since no fusion antigen is detected in the chase medium by



**Figure 1.** Construction of the Ly-6ED<sup>b</sup> fusion gene. (A) Strategy for construction of the fusion gene (see text). (B) The junction of the fusion protein. The linker amino acids and the restriction site are indicated.

immunoprecipitation (Fig. 2 A). To determine whether this fusion antigen is a GPI-anchored protein that utilizes some downstream GPI processing site or is an integral membrane protein, we subjected the Ly-6ED<sup>b</sup> to further analysis.

### The Fusion Protein Is an Integral Cell Surface Membrane Antigen

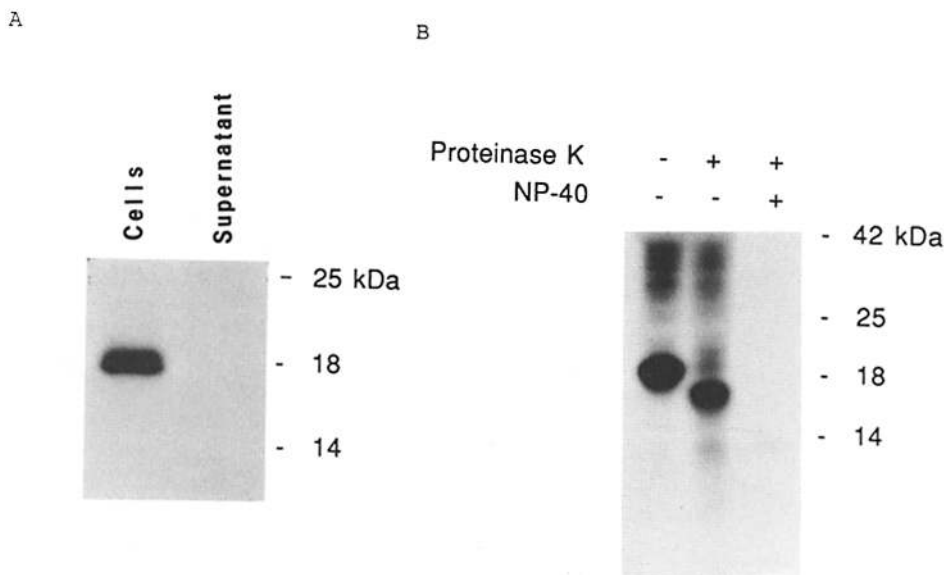
It has been shown that secretory proteins and portions of integral membrane proteins can be protected from protease digestion by microsomes (6, 10). This provides a convenient way to distinguish membrane bound from cytosolic proteins.

COS cells transfected with the Ly-6ED<sup>b</sup> plasmid were labeled with [<sup>35</sup>S]cysteine and microsomes were prepared by Dounce homogenization. After treatment with proteinase K, the remaining proteins were further immunoprecipitated and separated on SDS-PAGE. As indicated in Fig. 2 B, the fusion antigen was completely digested by proteinase K in the NP-40-permeabilized microsomes preparation. Treatment with proteinase K of the intact microsomes reduced the apparent mass of the fusion protein by ~2 kDa, indicating removal of ~20 amino acids from the exposed cytoplasmic region. This result shows that the fusion antigen is an integral membrane protein with a cytoplasmic tail.

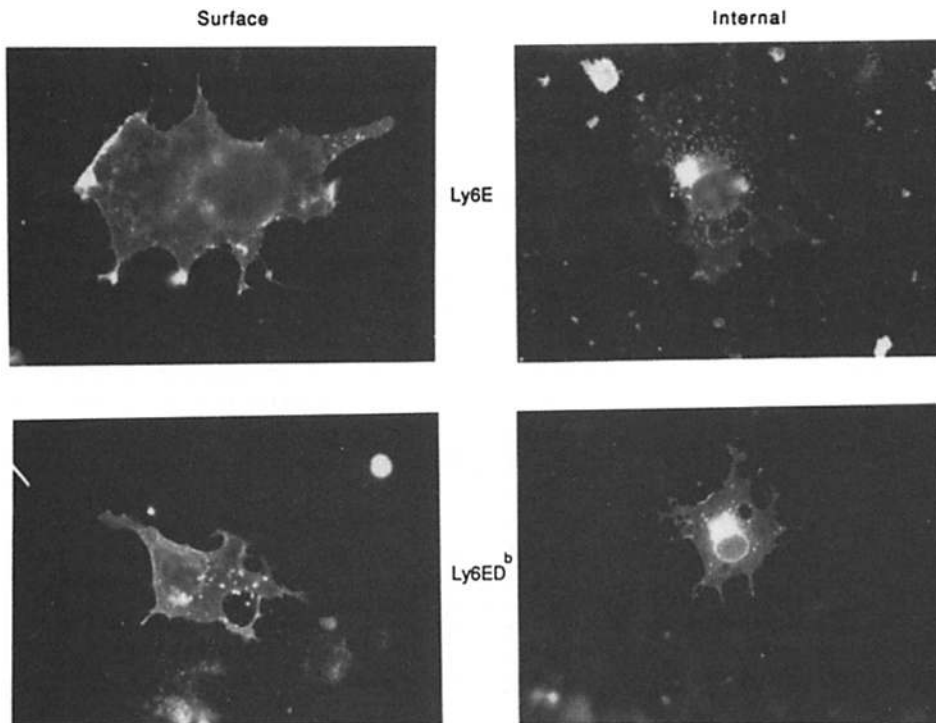
It has been shown previously that rat growth hormone, which is normally secreted, becomes an integral membrane protein when fused with the transmembrane and cytoplasmic domains of VSV G protein, but that the resultant hybrid protein is not transported to the cell surface (6). This transport failure can be corrected, however, by the introduction of an N-glycosylation site into the fusion protein (7). Like rat growth hormone, the Ly-6ED<sup>b</sup> antigen has no N-glycosylation sites in its sequence (20), but its successful expression as an integral transmembrane protein suggests that the Ly-6E extracellular domain contains signal(s) sufficient to direct cell surface transportation. To further demonstrate that the fusion protein was a cell surface antigen, we conducted indirect immunofluorescence staining experiments to determine whether this fusion protein is transported to the cell surface. As shown in Fig. 3, both the wild-type Ly-6E antigen and the fusion proteins were clearly detected intracellularly as well as on the surface of the transfected COS cell. The results demonstrate that the Ly-6ED<sup>b</sup> fusion antigen is successfully transported to the cell surface as is the GPI-anchored Ly-6E antigen.

### Ly-6ED<sup>b</sup> Is Not a GPI-linked Protein

To investigate whether there is any detectable Ly-6ED<sup>b</sup> protein anchored in the cell membrane by a glycosyl-phos-

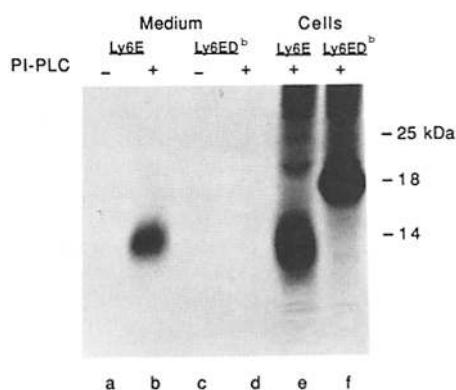


**Figure 2.** Expression of the Ly-6ED<sup>b</sup> antigen in COS cells. (A) COS cells were transiently transfected with plasmid DNA and assayed as described in Materials and Methods. Proteins from the chase medium or cell lysates were precipitated with the anti-Ly-6E antibody SK70.94 and subjected to electrophoresis. The antigen in each lane represents half of the protein precipitated either from cells or from medium of 3.5-cm dishes. (B) The sensitivity of Ly-6ED<sup>b</sup> antigen to proteinase K was examined in microsomes. After treatment with or without proteinase K, the antigens were precipitated by antibodies and separated on SDS-PAGE. The samples with or without proteinase K in the presence or absence of NP-40 are indicated.



**Figure 3.** Indirect immunofluorescence of wild-type Ly-6E antigen and Ly-6ED<sup>b</sup> fusion gene product. Internal and surface stains are indicated.

phatidylinositol linkage, the fusion protein was assayed for sensitivity to phosphatidylinositol specific phospholipase C (PI-PLC). We have shown previously that wild-type Ly-6E can be released from the surface of transfected COS cells by treatment with this enzyme (25). As shown in Fig. 4, the Ly-6E antigen on the COS cell surface is released into the medium by PI-PLC (lane *b*), whereas the fusion protein is resistant to the enzyme (lane *d*) despite the expression of equivalent amounts of protein by both gene constructs (lanes *e* and *f*). These results indicate that the fusion protein is not expressed as a GPI-anchored cell surface protein.



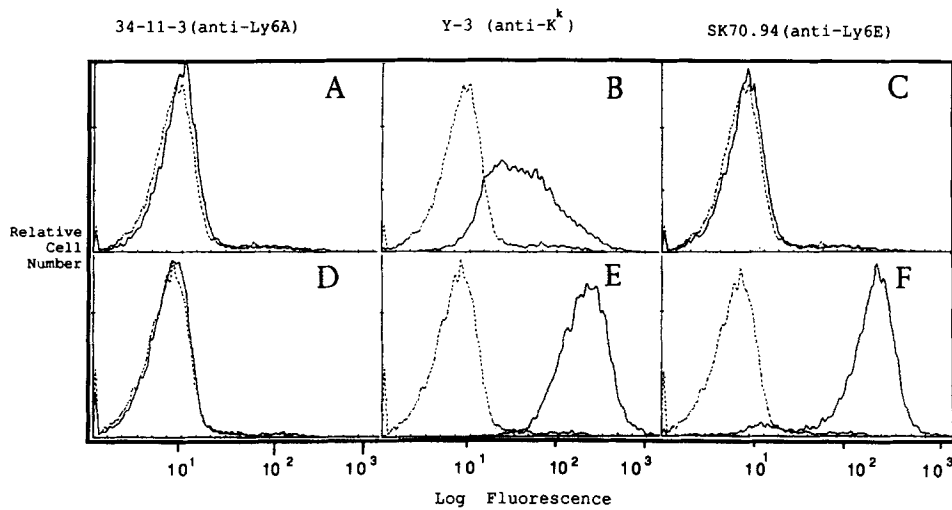
**Figure 4.** Sensitivity of wild-type Ly-6E antigen and Ly-6ED<sup>b</sup> antigen to PI-PLC. COS cells transfected with DNA were labeled for 2 h with [<sup>35</sup>S]cysteine. Cells were treated with PI-PLC for 2 h as described in Materials and Methods. The PI-PLC treated samples are indicated with (+), and the untreated with (-). The control was treated exactly the same except there was no enzyme in the medium. Antigens immunoprecipitated from either PI-PLC-treated cells or medium were separated by SDS-PAGE. Lanes *e* and *f* are immunoprecipitated samples from cell lysates.

#### **Expression of the Fusion Protein in a BW5147 Class "E" Cell Line**

Several somatic mutant cell lines of the AKR thymoma BW5147 have been reported, which are deficient in expression of Thy-1 and other GPI-anchored proteins on their surfaces (2, 3). In one of these, the class "E" mutant, it has been shown that the deficiency is due to the lack of biosynthesis of the glycolipid precursor for the GPI linkage (2). To study the behavior of Ly-6ED<sup>b</sup> in the class "E" mutant, we introduced the fusion gene into this cell line by electroporation. Expression of the antigen in one stable G418 resistant clone was analyzed by FACS<sup>®</sup> staining (Fig. 5). The Ly-6A antigen is not expressed on the surface of the class "E" mutant cell line (Fig. 5 *A*) despite the presence of high levels of Ly-6 mRNA (15), whereas BW5147 expresses very high levels of Ly-6A antigen on its surface. In contrast, this cell line exhibits normal surface expression of the integral membrane protein MHC class I K<sup>k</sup> (Fig. 5 *B*). Fig. 5 *C* shows that there is no cross-reactivity of anti-Ly-6E mAb to the class "E" cell line. When the class "E" mutant cell line is transfected with the Ly-6ED<sup>b</sup> gene, however, high levels of the fusion antigen are detected on the cell surface (Fig. 5 *F*). As expected, the transfectant does not express the endogenous GPI-anchored Ly-6A antigen (Fig. 5 *D*), but still expresses surface class I MHC K<sup>k</sup> (Fig. 5 *E*). These results demonstrate that the Ly-6ED<sup>b</sup> fusion gene can be expressed on the surface of lymphocytes as an integral transmembrane protein.

#### **Expression of Ly-6E, Ly-6ED<sup>b</sup>, and Class I D<sup>d</sup> Constructs in D10 Cells, a Type 2 T Helper Clone**

D10 is a conalbumin-specific T helper cell cloned from an AKR mouse that expresses high levels of Ly-6A (9, Fig. 6 *A*). There are two mAbs that distinguish the Ly-6A from its



**Figure 5.** Flow cytometry analysis of Ly-6ED<sup>b</sup> gene expression in transfected BW5147 class E mutant cell line. The samples for FACS staining were prepared as described (15) and run on a FACS 440<sup>®</sup>. The staining patterns of BW5147 class "E" mutant cell line using 34-11-3, Y-3, and SK70.94 are shown in A-C. The staining patterns of a stably transfected clone are shown in panels D-F. The dashed profiles indicate the staining with secondary antibody alone.

allelic form Ly-6E (see below). Three different constructs (Ly-6E, Ly-6ED<sup>b</sup>, and class I D<sup>b</sup>) were subcloned into the pcDL-SR $\alpha$  expression vector at the unique Eco RI cloning site (27) and transfected into D10 cells by electroporation. The G-418-resistant clones were then screened by FACS staining for the expression of the transfected genes. The parental D10 cells stained with 34-11-3 (anti-Ly-6A), SK70.94 (anti-Ly-6E), and H141-31 (anti-class I D<sup>b</sup>) are shown in Fig. 6 A-C, respectively. As expected, the anti-Ly-6E and anti-class I D<sup>b</sup> antibodies do not bind to D10 cells, whereas anti-Ly-6A antibody shows high levels of binding (Fig. 6 A). Fig. 6 D-F show the staining patterns of three individual D10 transfectants with anti-Ly-6E (D and E) or class I D<sup>b</sup> (F) mAbs (see above), respectively, and the expression levels of these transfected genes are similar in all these cases. In addition, these transfectants also express levels of the endogenous Ly-6A antigen which are comparable to that of parental D10 cells (data not shown).

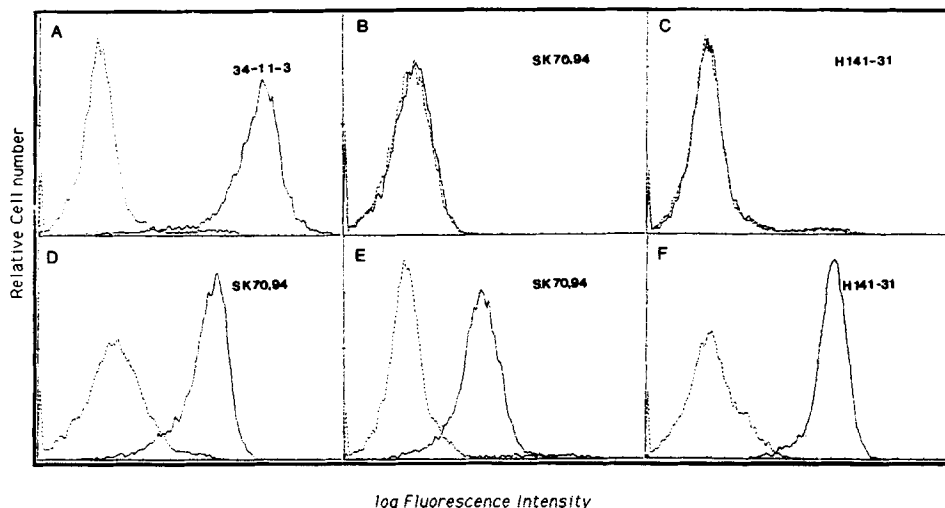
#### **D10 Cells Can Be Activated through the Ly-6A-mediated Activation Pathway**

We first measured the proliferation of D10 cells in response to

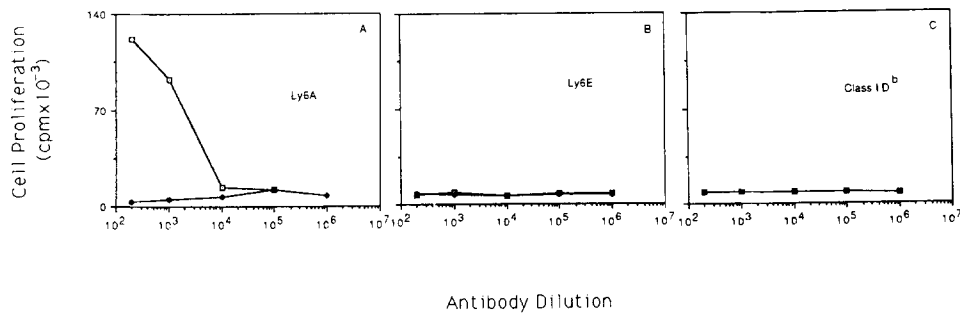
34-11-3, an anti-Ly-6A mAb. The results are shown in Fig. 7 A. Although there is no proliferation in the absence of rat anti-mouse immunoglobulin even at a very high concentration of antibody, the D10 cells proliferated vigorously after the Ly-6A molecules were cross-linked (Fig. 7 A). This result indicates that the crosslinking of Ly-6A antigen on the D10 cell surface is necessary to activate this T cell. This has been shown with the Ly-6A/E-mediated T cell activation in other systems, such as splenic T cells or T hybridomas (1, 23). Anti-Ly-6E and anti-class I D<sup>b</sup> (H141-31) antibodies have no effect on D10 cell proliferation either in the presence or absence of rat anti-mouse immunoglobulin (Fig. 7 B and C).

#### **Transmembrane-anchored Ly-6E Is Unable to Stimulate D10 Cell Proliferation**

To investigate the requirement of the GPI anchor in Ly-6A/E mediated T cell activation, the transmembrane Ly-6ED<sup>b</sup> fusion antigen was expressed on the surface of D10 T helper cell by gene transfection (see Fig. 6). As shown in Fig. 8 A-C, the Ly-6E, Ly-6ED<sup>b</sup>, and class I D<sup>b</sup> transfectants are fully responsive to antibody against the endogenous GPI-anchored Ly-6A antigen. The activation of D10 cell and its



**Figure 6.** Flow cytometry analysis of the expression of Ly-6E, Ly-6ED<sup>b</sup>, and the MHC class I D<sup>b</sup> genes in D10 cells. The samples were prepared as described (15) and analyzed using a FACS 440<sup>®</sup>. A-C show the staining patterns of D10 cells with three different mAbs as indicated. D and E show the staining patterns of Ly-6E and Ly-6ED<sup>b</sup> transfected D10 clones with the SK70.94 antibody, respectively. F shows the staining pattern of the class I D<sup>b</sup> D10 transfectant stained with H141-31. The staining with the secondary antibody alone are shown with the dashed profiles.

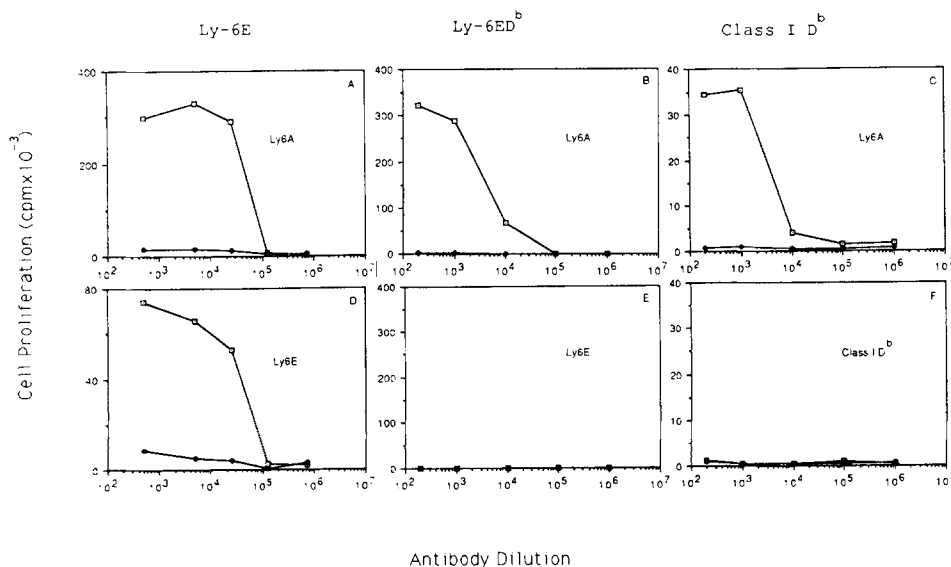


**Figure 7.** Proliferative responses of D10 cells mediated by Ly-6A. Resting D10 cells were treated with mAbs: (A) anti-Ly-6A(34-11-3) (B) anti-Ly-6E(SK70.94) and (C) anti-class I D<sup>b</sup>(H141-31) as described in Materials and Methods. The presence or absence of rat anti-mouse IgG are indicated by the symbols  $\square$ - and  $\blacksquare$ -, respectively.

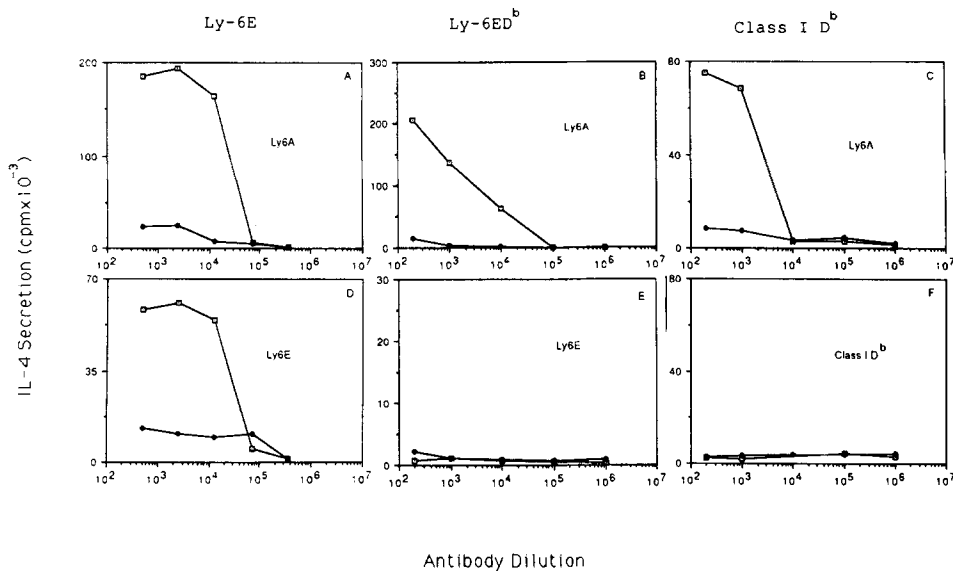
transfectants mediated by the endogenous Ly-6A antigen does not differ significantly. There is variation in the absolute proliferative response of individual T cell clones which may be due to the length of time that has elapsed since the last stimulation with antigen-pulsed spleen cells. However, when antibodies directed against the transfected gene products were used, only the transfectant that expresses the GPI-anchored Ly-6E antigen can be stimulated to proliferate (Fig. 8 D), demonstrating that the activation capability of the GPI-anchored Ly-6E antigen was not affected by the gene transfection. However, the Ly-6ED<sup>b</sup> transfectant which expresses an integral membrane anchored Ly-6E antigen did not respond to the anti-Ly-6E antibody as shown in Fig. 8 E, although this antibody is fully capable of recognizing the fusion protein (see Fig. 6). As expected, anti-class I D<sup>b</sup> antibody did not activate the D10 transfectant expressing class I D<sup>b</sup> protein (Fig. 8 F). This is consistent with the previous finding that in the murine system class I antigens do not induce T cell activation (22, 24). These results demonstrate that the GPI anchor of the Ly-6A/E antigen is critical for T cell activation mediated by Ly-6A/E molecules.

### Only GPI-anchored Ly-6A/E Can Induce Lymphokine Secretion But Not the Integral Membrane Form of Ly-6ED<sup>b</sup> Fusion Antigen

D10 cells are a type 2 T helper clone which can be induced to release IL 4 by antigen or by mAbs to the T cell receptor or CD3 molecules (9). It can also be stimulated to secrete IL 4 by cross-linking Ly-6A on the surface (data not shown). To examine the importance of the GPI anchor of the Ly-6A/E antigen in inducing D10 cells to secrete IL 4, we assayed lymphokine production in the above transfectants. The lymphokine secretion can be induced through the endogenous GPI-anchored Ly-6A antigen as shown in Fig. 9 by the induction of CTLL cell proliferation as indicated in A (Ly-6E transfectant), B (Ly-6ED<sup>b</sup> transfectant) and C (class I D<sup>b</sup> transfectant). However, while the GPI-anchored Ly-6E transfectant can be stimulated to release IL 4 (Fig. 9 D) with anti-Ly-6E antibody, this is clearly not the case with the transmembrane anchored Ly-6ED<sup>b</sup> transfectant (Fig. 9 E). Likewise, the class I D<sup>b</sup> transfectant does not release lymphokine when crosslinked with anti-class I D<sup>b</sup> antibody



**Figure 8.** Stimulation of D10 cells mediated by endogenous Ly-6A, transfected Ly-6E, Ly-6ED<sup>b</sup>, and class I D<sup>b</sup> gene products. The transfected cells were treated with antibody to the endogenous Ly-6A or the transfected gene products and cultured at 37°C in the presence  $\square$ - or absence of  $\blacksquare$ - rat anti-mouse IgG F(ab)<sub>2</sub> as described in Materials and Methods. (A and D) Ly-6E transfectant; (B and E) Ly-6ED<sup>b</sup> transfectant; (C and F) class I D<sup>b</sup> transfectant. The proliferative response to the endogenous Ly-6A antigen of the three transfectants is indicated in A-C. The proliferative response mediated by the transfected gene products are indicated in D-F. In E, the [<sup>3</sup>H]thymidine incorporation was between 300 and 700 cpm for all antibody concentration tested. The monoclonal antibodies used are the same as described in Fig. 7.



**Figure 9** Lymphokine secretion induced by GPI-anchored Ly-6E but not by transmembrane anchored Ly-6E. Lymphokine containing supernatant from the mAb-stimulated D10 cells was prepared and assayed as described in Materials and Methods. The symbols  $\square$  and  $\blacksquare$  shows the presence or absence of rat anti-mouse IgG. *A-C* show the CTLL proliferation stimulated by the lymphokine secreted from the Ly-6E, Ly-6ED<sup>b</sup> and class I D<sup>b</sup>-transfected D10 cells mediated through the endogenous Ly-6A antigen respectively. *D-F* show the CTLL proliferation stimulated by the lymphokine secreted from the transfectants activated through their transfected gene products as indicated.

(Fig. 9 *F*). Our results demonstrate that a GPI anchor is necessary for Ly-6A/E mediated lymphokine secretion from D10 T cells.

### Discussion

To investigate the significance of the GPI anchor of the Ly-6E molecule, the wild-type Ly-6E antigen has been converted into a transmembrane protein. This was accomplished by replacing the hydrophobic tail with the transmembrane and cytoplasmic domains of the MHC class I D<sup>b</sup> protein. When expressed in COS cells, the fusion protein has been shown to be an integral cell surface membrane protein by the PI-PLC digestion assay, the microsomal protection assay and the indirect antibody staining assay. Furthermore, this protein can be expressed on the surface of the BW5147 class "E" mutant cell line which is defective in the biosynthesis of GPI-modified proteins. To study the function of the altered membrane anchor of the Ly-6E molecule, this fusion gene as well as GPI anchored Ly-6E gene have been transfected into a T cell clone. While the GPI-anchored Ly-6E antigen, as well as the endogenous GPI-anchored Ly-6A antigen, can initiate T cell activation when cross-linked, the transmembrane-anchored Ly-6ED<sup>b</sup> antigen was unable to mediate such T cell activation.

We have previously undertaken a systematic approach to define the signal requirement for the GPI linkage of the Ly-6E molecule by site-directed mutagenesis of the carboxy-terminal sequence (25). While specific point mutations and deletions were found to partially inhibit or abolish the GPI processing signals and result in secreted products, a transmembrane form of the Ly-6E antigen was not created. In addition, one mutant with an overall structure analogous to the Ly-6ED<sup>b</sup> fusion protein in that it contains the putative GPI processing site followed by a stretch of hydrophobic sequence and a charged cytoplasmic tail, did not exhibit integral membrane expression. This suggests that the signals contained in the hydrophobic segments in transmembrane and GPI-anchored proteins may be different. These differences may be distinguished by sorting proteins or by the GPI

processing apparatus resulting in transmembrane protein expression or GPI-anchored protein expression. Assuming that there are two different signals for this different expression, the successful expression of the Ly-6ED<sup>b</sup> fusion protein as an integral membrane protein suggests that the signals for transmembrane expression from the D<sup>b</sup> protein are dominant in the fusion protein.

Transmembrane signaling through a conventional transmembrane receptor is thought to be carried out by the transmembrane/cytoplasmic domain (8). This domain is absent from GPI-anchored molecules, raising the question of how GPI-anchored molecules deliver signals across membrane. Most interestingly, our present report provides a direct comparison of the relative capacities of the GPI-anchored Ly-6E and transmembrane Ly-6ED<sup>b</sup> molecules in the activation of a T cell clone to proliferate in vitro and release lymphokines. Our results show that activation of T cells through Ly-6A/E antigen is completely dependent on the presence of the GPI anchor, since the integral membrane-anchored Ly-6E antigen is unable to mediate T cell activation. This requirement of a GPI anchor for T cell activation suggests that the GPI moiety itself is involved in signal transduction and is more than a passive attachment point. In this regard, similar results have been recently observed in studies of transgenic animals with another T cell surface protein, the Qa-2 antigen (22).

The mechanism whereby the GPI moiety delivers signals across membrane is unknown. One possibility is that the GPI-anchored proteins may interact with other transmembrane proteins to transduce a signal across the membrane. This idea is supported by the finding that Ly-6 mediated T cell activation requires the expression of the T cell receptor/CD3 complex (1, 26). In addition, it has been shown that T cells and T cell hybridomas with reduced or inhibited Ly-6A protein expression exhibit a decreased antigen response (5, 30). Such interaction of the GPI-linked Ly-6E molecule with other transmembrane molecules may be directly mediated by the GPI-anchor, or may be influenced by the increased lateral mobility which the GPI-anchor provides (4). Confirmation of a physical interaction between Ly-6 and

TCR/CD3 complex or other molecules will require more direct evidence. However, no association of Ly-6A and either TCR/CD3, CD4 or CD45 molecules has been observed in cocapping experiments with cross-linking antibodies (Su, B., and A. L. M. Bothwell, unpublished observations).

A second possibility is that the GPI anchor may play a direct role in signal transduction during Ly-6A/E mediated T cell activation. A recent study by Rock et al. (23) shows that the activation of T cells mediated by Ly-6A requires the internalization of the Ly-6A-antibody complex but it is not the case for T cell receptor-mediated cell activation. From these results, the authors suggest that T cell activation mediated by Ly-6 may utilize a different pathway from that mediated by the T cell antigen receptor. It is possible that the GPI-anchor or its degradation products may provide a second message in this putative activation pathway (4).

Our current study strongly suggests that signaling through Ly-6A/E in T cell activation is dependent on the presence of an intact GPI anchor. Whether the GPI anchor associates with another membrane protein to transduce a signal across the cell membrane or is itself involved in signal transduction is unknown. The transmembrane Ly-6 could serve to inhibit signaling through Ly-6 by competition for its unknown ligand. Thus, the stable D10 transfectants described in this paper that overexpress the transmembrane form of Ly-6E should be useful for studies of the role of Ly-6E in the antigen-specific T cell response.

We thank Dr. Martin Low for his generous gift of PI-PLC, Dr. Jack Rose for the transient expression vector JC119R, Margot Bridgett for preparing antibodies and Tom Taylor for flow cytometry analysis. We also wish to thank William Philbrick, Yang Liu, Umberto Dianzani and other members in the Bothwell laboratory for comments on both experimental procedures and the manuscript.

This work was supported by grant GM-40924 from the U.S. Public Health Service. A. Bothwell is an Associate Investigator of the Howard Hughes Medical Institute.

Received for publication 28 December 1989 and in revised form 30 October 1990.

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