Determination of the non-ionic detergent insolubility and phosphoprotein associations of glycosylphosphatidylinositol-anchored proteins expressed on T cells

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Glycosylphosphatidylinositol (GPI)-anchored proteins are poorly solublized in non-ionic detergents such as Triton X-100 and Nonidet P40, but are easily solublized by detergents with high critical micelle concentrations such as octylglucoside. This solubility profile has been suggested to be due to the localization of GPI-anchored proteins to lipid microdomains rich in cholesterol and sphingolipids. Additionally, GPI-anchored proteins expressed on haemopoietic cells have been shown to associate with src-family tyrosine kinases and heterotrimeric G proteins. Despite these observations, the non-ionic detergent insolubility of GPI-anchored proteins on haemopoietic cells has not been quantified nor has a relationship between the non-ionic detergent insolubility of these proteins and their association with signal-transduction molecules been identified. Here we show that GPI-anchored proteins found on T-cell tumours and activated T cells, although significantly more insoluble then transmembrane proteins, are not uniform in their detergent insolubility. Whereas CD59 was between 4% and 13% soluble, CD48 was between 13% and 25% soluble, CD55 was between 20% and 30% soluble, and CD109 was between 34% and 75% soluble. The ability of these GPI-anchored proteins to associate with phosphoproteins was correlated with their detergent insolubility: the more detergent-insoluble that a GPI-anchored protein was, the greater the level of phosphoprotein associations. These experiments reveal a relationship between non-ionic detergent insolubility and association with signal-transduction molecules and suggest a cause-and-effect relationship between these two properties. In total, these experiments support the hypothesis that the association of GPI-anchored proteins with signalling molecules is due to their sorting to lipid microdomains.

INTRODUCTION

Many glycosylphosphatidylinositol (GPI)-anchored proteins expressed on lymphocytes have been shown to transduce intracellular signals, leading to tyrosine phosphorylation, calcium mobilization, interleukin-2 production and cellular proliferation [1–8]. These proteins have also been shown to associate with src-family kinases and heterotrimeric G proteins [1,8–13]. In contrast to transmembrane receptors, GPI-anchored proteins are attached to the cell surface by virtue of their GPI anchor and do not have transmembrane-domain/cytoplasmic-tail amino acids. Thus, the mechanism by which GPI-anchored proteins are associated with intracellular messengers is different than other integral membrane proteins with more conventional transmembrane and intracellular protein domains.

GPI-anchored proteins are relatively insoluble in cold non-ionic detergents, i.e. Triton X-100 and Nonidet P40 (NP40), but are soluble in detergents with high critical micelle concentrations (CMCs), i.e. CHAPS and octylglucoside [14–20]. This solubility profile has been determined to result directly from the localization of GPI-anchored proteins to membrane microdomains rich in cholesterol and sphingolipids [21–23]. In some cell types (e.g. endothelial cells) there are at least two different types of lipid microdomain [24,25]: those that contain GPI-anchored proteins and those that are organized into caveolae, which are striated plasmalemmal vesicles organized and marked by the protein caveolin [26–31]. The situation in haemopoietic cells is greatly simplified by the fact that these cells do not express caveolin and do not have morphological caveolae [12,32–33]. The lipid microdomains of haemopoietic cells are likely to be free-floating lipid rafts, lacking the higher level of membrane organization found in caveolae.

It has been proposed [13,34] that the association of the GPI-anchored protein with signal-transduction molecules is dependent on the co-localization of the GPI-anchored proteins and dual-acylated signalling molecules to the aforementioned lipid microdomains. A prediction based on this model is that there is a relationship between the non-ionic detergent insolubility of GPI-anchored proteins and their ability to associate with cytoplasmic messengers.

Whereas many studies report the non-ionic detergent insolubility of GPI-anchored proteins ([14–20] and many others), quantification of this insolubility and direct comparison of the insolubility of multiple GPI-anchored proteins has only been performed with GPI-anchored proteins expressed on kidney microvillus membranes [14].

Here we quantified and compared the Triton X-100 insolubility of six different integral membrane proteins (two transmembrane and four GPI-anchored) on two T-cell tumour lines and activated T-cell blasts, and we show a range of solubilities for different proteins. We also performed analysis of the phosphoproteins associated with each GPI-anchored protein

Abbreviations used: GPI, glycosylphosphatidylinositol; CMC, critical micelle concentration; PHA, phytohaemagglutinin; PEG, poly(ethylene glycol); NP40, Nonidet P40; mAb, monclonal antibody; VLA, very late antigen.

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from each cell line and reveal a correlation between the Triton X-100 insolubility of a particular species and its association with phosphoproteins. Taken in total these studies support a model in which GPI-anchored proteins are associated with signal-transduction molecules by virtue of the co-localization of GPI-anchored proteins and signal-transduction molecules to detergent-insoluble lipid microdomains.

EXPERIMENTAL

Cells

HSB and HPB-ALL cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and penicillin/streptomycin in 5% CO_2 at 37 °C. Human T lymphocytes were obtained from leukopaks (discarded leukocytes from platelet donations). Cells were fractionated on Ficoll–Hypaque gradients, washed, treated with Tris-buffered NH_4Cl to eliminate red blood cells, washed and passed through nylon wool. To activate the T cells, cells were incubated with phytohaemagglutinin (PHA, 10 μg/ml) at a concentration of 10^6 cells/ml in RPMI 1640 and 10% fetal calf serum at 37 °C for 3 days prior to assay.

Antibodies and flow cytometry

The following antibodies were used in these studies: MEM-43 (anti-CD59; Caltag, San Francisco, CA, U.S.A.); IA10 [anti-DAF (decay accelerating factor); CD55, gift of Dr. V. Nussenzweig, New York University, New York, U.S.A.]; IF7 (anti-DAF; CD55, gift of Dr. J. Bergelson, Dana-Farber Cancer Inst., Boston, MA); 1B3 (anti-CD109; gift of Dr. I. Bernstein, Fred Hutchinson Cancer Center, Seattle, WA, U.S.A.); LDA-1 (anti-CD109; gift of Dr. N. Suciu-Foca, Columbia University, New York, U.S.A.); D2 (anti-CD109 [35]); W632 [anti-(class-I MHC); ATCC, Rockville, MD, U.S.A.]; 4G10 (anti-phosphotyrosine; gift of Dr. B. Druker, Oregon Health Sciences Center, Portland, OR, U.S.A.); anti-fyn antiserum (gift of Dr. C. Rudd, Dana-Farber Cancer Inst.); anti-lck antiserum (gift of Dr. C. Rudd); DE9 [anti-(very late antigen-2, or VLA-2, β chain); gift of Dr. J. Bergelson]; MEM-102 (anti-CD48; Biosource International, Camarillo, CA, U.S.A.); and MOPC 195 (negative control; Sigma, St. Louis, MO, U.S.A.). For immunofluorescence analysis $(0.5–1) \times 10^6$ cells were incubated for 45 min with saturating concentrations of monclonal antibody (mAb) at 4 °C, washed, incubated for 45 min with FITC-conjugated goat anti-mouse Ig (Sigma), washed, fixed in 1% formalin and analysed on a FACSCAN (Becton Dickinson, Mountain View, CA, U.S.A.).

Immunoprecipitation, *in vitro* kinase assay and re-immunoprecipitation

Cells were washed three times in cold PBS (or Hanks balanced salt solution) and were lysed on ice for 30 min in lysis buffer [0.5% Triton X-100 (or NP40)/300 mM NaCl/50 mM Tris/HCl (pH 7.6)/0.15 units/ml aprotinin (Sigma)/5 mM EDTA/1 mM Na_3VO_4/10 μg/ml leupeptin/1 mM PMSF/10 mM iodoacetamide]. Insoluble debris were removed by microcentrifugation (5700 ***g***) for 20 min and the lysates were precleared (30 min each) with 100 μl (10% w/v) of rabbit anti-mouse coated Protein A–Sepharose beads (1 mg/ml) followed by 200 μl of (10% w/v) Protein A–Sepharose beads. The lysates were then incubated for 2 h at 4 °C with mAbs bound previously to Protein A–Sepharose beads. After 2 h the beads were washed four times in lysis buffer and once in kinase buffer [25 mM Hepes/1 mM $MnCl_2$/100 μM Na_3VO_4]. The immunoprecipitates were then resuspended in 50 μl of kinase buffer with 20 μCi [γ-^{32}P]ATP (New England Nuclear, Boston, MA, U.S.A.) and incubated for 15 min at room temperature. The samples were then washed four times in lysis buffer with 15 mM EDTA. Samples were either eluted in 0.5% SDS at 70 °C for 3 min or boiled in 1% SDS for 5 min and diluted 10-fold with cold lysis buffer. The eluate was either subjected directly to SDS/PAGE analysis or was subjected to re-immunoprecipitation with various mAbs or polyclonal antisera (see Figures) and 20 μl of Protein A–Sepharose beads for 2 h at 4 °C. Re-immunoprecipitated samples were washed four times in lysis buffer, resuspended in reducing Laemmli sample buffer, boiled and subjected to electrophoresis through a 10% SDS/PAGE gel. In the experiments reported here 0.5% detergent was used in the lysis buffer, though similar results were obtained with 1% detergent. The choice of 0.5% detergent was made for consistency with our own previous studies [13,36] and those of others [10].

Iodination and solubility assay

Cells were iodinated via lactoperoxidase/glucose oxidase-catalysed iodination [36] and then resuspended in buffer A [25 mM Mes/150 mM NaCl (pH 6.5)]. To this an equal volume of the same buffer with 2% Triton X-100 (or NP40) and 2 mM PMSF (final concentration of Triton X-100 was 1%) was added, and lysates were incubated on ice for 30 min. Insoluble fractions were pelleted in a microcentrifuge (5700 ***g***) for 20 min at 4 °C. Supernatants were removed (soluble fraction) and the insoluble pellet was resuspended in buffer B [1% Triton X-100/10 mM Tris/HCl (pH 7.6)/500 mM NaCl/2 mM PMSF/60 mM octylglucoside (Sigma)] for 30 min on ice. Debris was pelleted in a microcentrifuge (5700 ***g***) for 20 min at 4 °C, and the supernatant was removed (insoluble fraction). Preclearing and immunoprecipitation were performed as described for *in vitro* kinase assays. Immunoprecipitates were washed four times in lysis buffer, resuspended in reducing Laemmli sample buffer, boiled and subjected to electrophoresis through a 10% SDS/PAGE gel. Quantification was performed by exposing gels to a PhosphoImaging screen (Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.) with subsequent analysis on a PhosphoImager using ImageQuant software (Molecular Dynamics Inc.). In the experiments reported here, 1% detergent was used in the lysis buffer, though similar results were obtained with 0.5% detergent (both concentrations are significantly above the CMC of Triton X-100). The choice of 1% detergent was made for consistency with our own previous studies [36] and those of others [16–19].

RESULTS

GPI-anchored proteins expressed on T cells have a wide range of Triton X-100 solubilities

Most studies on the capacity of GPI-anchored proteins to initiate signal transduction and to associate with signalling molecules have been performed in haemopoietic cells, whereas studies that have measured the detergent insolubility of these proteins have been performed on non-haemopoietic cell types [1–20]. Since it has been hypothesized that both the detergent insolubility and the association with signalling molecules are due to localization of GPI-anchored proteins to specialized membrane microdomains rich in cholesterol and sphingolipids [13,34], it was of interest to us to quantify and compare the Triton X-100 solubility of a multiple GPI-anchored proteins from T-cell tumours and activated T cells.

HPB-ALL, HSB and activated T cells were iodinated and solublized in ice-cold Triton X-100 lysis buffer, the insoluble

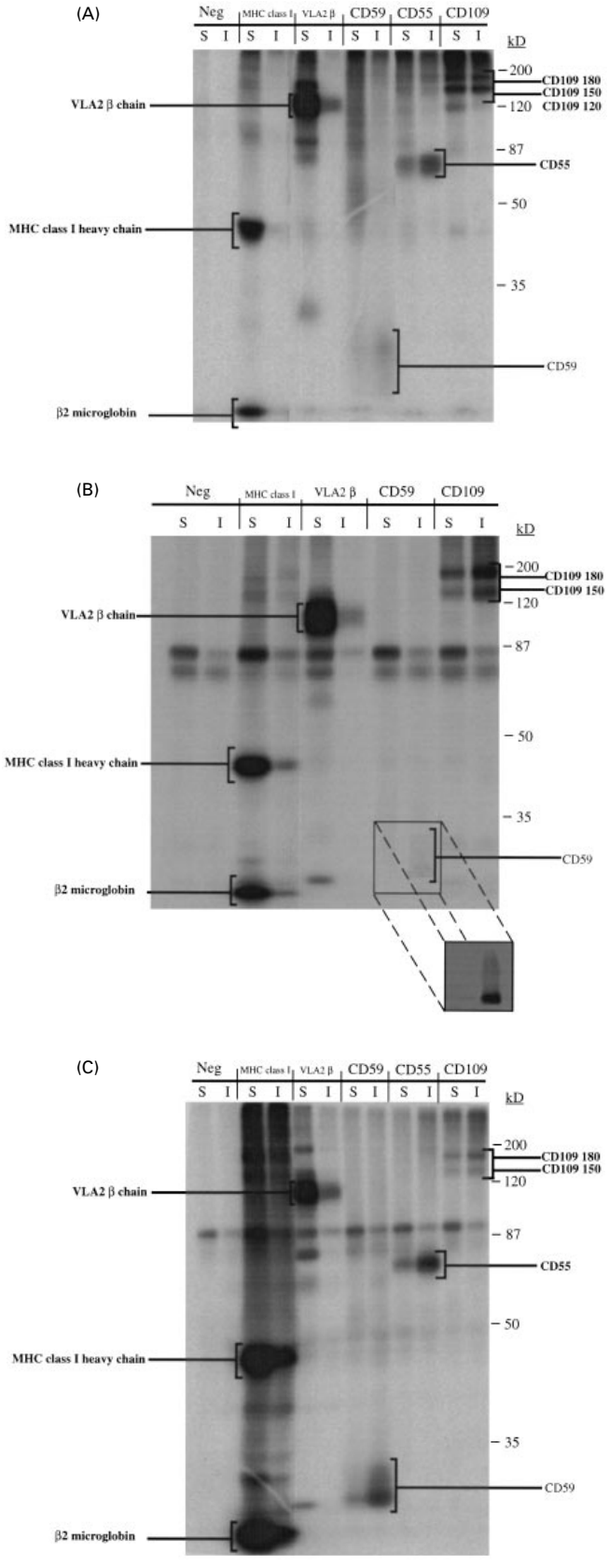

Figure 1 Solubility of integral membrane proteins expressed in T-cell lines and activated T cells

(**A**) Representative solubility analysis of integral membrane proteins expressed on HPB-ALL ALL cells. HPB-ALL cells were iodinated, cells were lysed in Triton X-100 (or NP40) lysis buffer, insoluble material was pelleted and the supernatant removed (soluble fraction, S). The insoluble pellet was then resolubilized in Triton X-100 lysis buffer plus octylglucoside (insoluble fraction, I). Each fraction (S and I) was subjected to immunoprecipitation with the mAbs (see below). (**B**) Representative solubility analysis of integral membrane proteins expressed on HSB cells. HSB cells were iodinated, lysed and treated as for HPB-ALL cells. The highlighted region of the gel was too faint to see with the exposure time that was optimal for the rest of the gel, so a longer exposure of this region is shown. (**C**) Solubility of integral membrane proteins expressed on activated T cells. Activated T cells were treated as for HPB-ALL cells. The CD109 120 kDa species, while not evident in the gel (**C**), could be seen on longer exposures and was present on the phosphoimager. In each experiment (**A**, **B** and **C**) both S and I fractions were subjected to immunoprecipitation with the following mAbs: MOPC (negative control), W632, DE9, MEM-43, IA10 (except for HSB cells which do not express CD55) and 1B3; the immunoprecipitates were resolved by 10% SDS/PAGE. Each specific immunoprecipitated species is indicated by brackets.

Table 1 Quantification of the solubility of integral membrane proteins expressed on T-cell lines and PHA-activated T-cell blasts

The experiments shown in Figure 1 and other similar experiments were quantified by exposing gels to a phosphoimaging screen followed by quantification using ImageQuant software. The data are represented as the percentage of Triton soluble [(soluble/soluble + insoluble) × 100] ± S.D. For each GPI-anchored antigen, $n = 3$; for transmembrane antigens, $n = 4$; for all antigens on activated T-cell blasts, $n = 1$.

		% Triton X-100 soluble		
Membrane attachment	Molecule	HPB-ALL	HSB	PHA blast
Transmembrane	VLA-2 β-chain	97.0 ± 2.6	91.1 ± 4.9	94.6
	Class I MHC	94.0 ± 6.6	96.5 ± 3.5	97.0
GPI-anchored	CD59	3.6 ± 3.4	10.5 ± 2.6	13.3
	CD109 180 kDa	49.3 ± 4.0	33.8 ± 11.3	37.1
	CD109 150 kDa	48.5 ± 3.1	34.0 ± 11.0	49.8
	CD109 120 kDa	70.3 ± 8.9	50.5 ± 2.1	45.4
	CD55	30.1 ± 2.3	Not expressed	20.2
	CD48	24.8 ± 0.8	12.8 ± 3.5	Not determined

material was pelleted and the supernatant removed (soluble fraction). The Triton-insoluble pellet was then resolubilized in Triton X-100 plus 60 mM octylglucoside, and the supernatant from this fraction was collected (insoluble fraction). The lysates were subjected to immunoprecipitation with various mAbs, as indicated in Figure 1, and resolved by SDS/PAGE. After exposure to film, the gels were exposed to a phosphoimaging screen and the amount of specific immunoprecipitated material in each lane was quantified (Table 1). As revealed in Figure 1A and Table 1, transmembrane proteins (MHC Class I and VLA-2 β-chain) expressed on HPB-ALL cells were well-solublized by Triton X-100 (95–100%), whereas GPI-anchored proteins (CD59, CD55 and CD48) were relatively insoluble in Triton X-100: 4% of CD59, 30% of CD55 and 25% of CD48 were Triton X-100-soluble. In contrast to the other GPI-anchored proteins, CD109 was between 49–70% soluble (depending on the form of the protein). The 180 and 150 kDa forms were both approx. 50% soluble in Triton X-100, whereas the 120 kDa form was 70% soluble in this detergent.

Like the proteins expressed on HPB-ALL cells, the transmembrane proteins expressed on HSB cells (Figure 1B, Table 1) were almost completely soluble in Triton X-100 (91–97%), whereas the GPI-anchored proteins showed a range of Triton X-100 solubilities: 11% for CD59; 13% for CD48; 34% for CD109 180 and 150 kDa forms; and 51% for CD109 120 kDa form. Analysis of the Triton X-100 solubility of proteins expressed on activated T cells (Figure 1C, Table 1) showed that whereas the MHC class I and VLA-2 β-chain transmem-

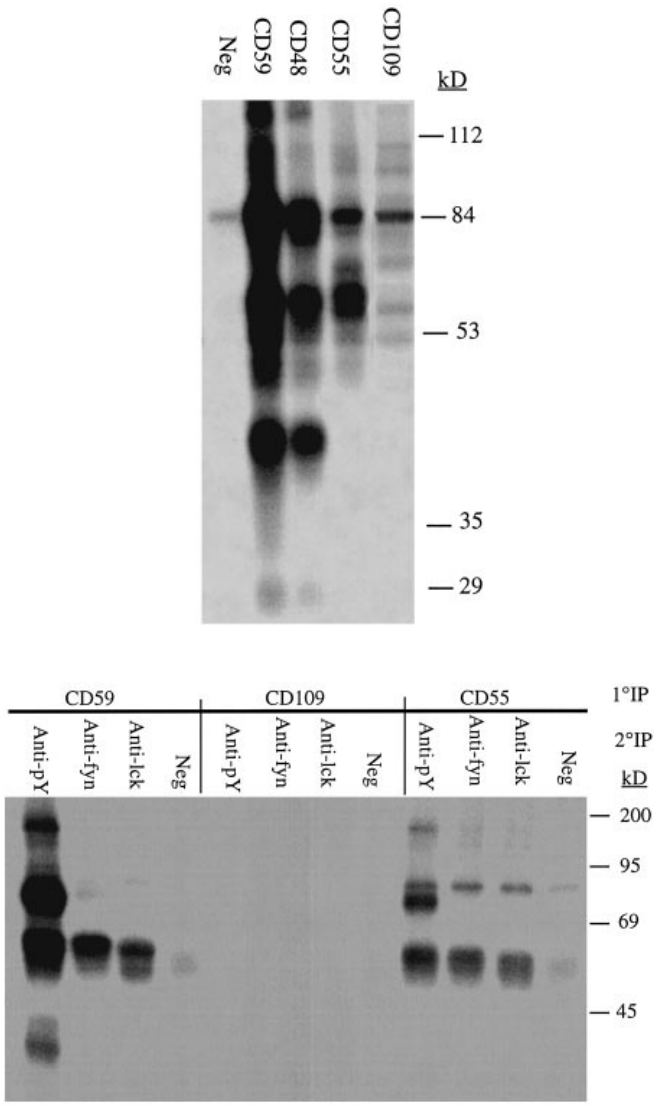

Figure 2 Analysis of kinases and phosphoproteins associated with GPI-anchored proteins expressed on HPB-ALL cells

(Upper panel) *In vitro* kinase assays of immunoprecipitates of GPI-anchored proteins expressed on HPB-ALL cells. HPB-ALL cells were lysed in NP40 (or Triton X-100) lysis buffer and the lysates were subjected to immunoprecipitation with the following mAbs: MOPC (negative control), MEM-43, MEM-102, IA10 and 1B3. The immunoprecipitates were washed, subjected to *in vitro* kinase assays as described in Materials and methods and resolved by 10% SDS/PAGE. (Lower panel) Analysis of the products labelled in the *in vitro* kinase assays performed on immunoprecipitates (1° IP) of GPI-anchored proteins expressed on HPB-ALL cells. The products of the CD59, CD109 and CD55 *in vitro* kinase assays were eluted from the immunoprecipitating beads and subjected to re-immunoprecipitation (2° IP) with either preimmune rabbit serum (negative control), anti-lck or anti-fyn kinase antisera, or with 4G10 anti-phosphotyrosine (anti-pY) mAb. The immunoprecipitates were washed and resolved by 10% SDS/PAGE.

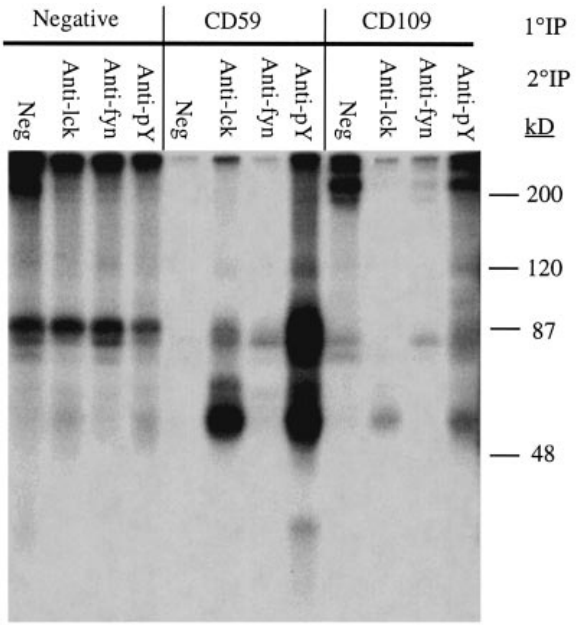

Figure 3 Analysis of kinases and phosphoproteins associated with GPI-anchored proteins expressed on HSB cells

The products of the CD59 (MEM-43), CD109 (1B3) and negative control (MOPC) *in vitro* kinase assays (1° IP) were eluted from the immunoprecipitating beads and were subjected to re-immunoprecipitation (2° IP) with either preimmune rabbit serum (negative control), anti-lck and fyn kinase antisera, or with 4G10 anti-phosphotyrosine (anti-pY) mAb. The immunoprecipitates were washed and resolved by 10% SDS/PAGE.

brane proteins were very soluble in Triton X-100, the GPI-anchored proteins were much less soluble: 13% for CD59; 20% for CD55; 37% for CD109 180 kDa; 45% for CD109 120 kDa form; and 50% for CD109 150 kDa form. Interestingly, of the GPI-anchored proteins, CD59 was the least soluble in all the cells tested (4–13%), whereas CD109 was the most soluble (34–70%). In all experiments performed (three times each for GPI-anchored proteins expressed on HPB-ALL and HSB cells, once for activated T cells), the non-ionic detergent insolubility of the GPI-anchored proteins was revealed not to be a uniform characteristic but varied from protein to protein.

Phosphoprotein associations vary with different GPI-anchored proteins

Since our investigation of the non-ionic detergent solubility of GPI-anchored proteins revealed that this was not a uniform characteristic, we next determined whether the phosphoprotein associations also varied between different GPI-anchored proteins. In order to visualize the association of phosphoproteins with GPI-anchored molecules, we took advantage of the fact that GPI-anchored proteins are associated with src-family kinases [1,10,12,13,36], which autophosphorylate and phosphorylate other proteins found in complexes with the GPI-anchored proteins *in vitro* [1,10,12,13,36] (other techniques, i.e. immunoblotting, are not sensitive enough to detect phosphoprotein/GPI-anchored protein associations). Lysates of HPB-ALL, HSB and activated T cells were subjected to immunoprecipitation with mAbs recognizing the GPI-anchored proteins CD59, CD48, CD55 or CD109, or with a negative control antibody. The immunoprecipitates were then subjected to *in vitro* kinase assays and the labelled products were resolved by SDS/PAGE. The results of the *in vitro* kinase assays performed on immunoprecipitates of GPI-anchored proteins expressed on HPB-ALL cells are shown in Figure 2. In these cells both CD59 and CD48 co-immunoprecipitated with three major phosphoprotein bands migrating at 85, 50–60 and 40 kDa respectively. This pattern of

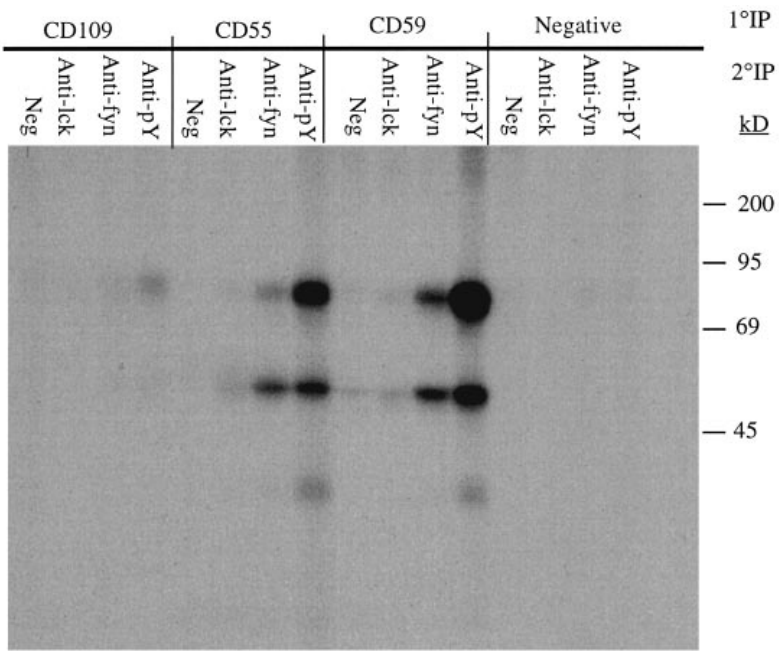

Figure 4 Analysis of kinases and phosphoproteins associated with GPI-anchored proteins expressed on activated T cells

The products of the CD109 (1B3), CD55 (IA10), CD59 (MEM-43) and negative control (MOPC) *in vitro* kinase assays (1° IP) were eluted from the immunoprecipitating beads and were subjected to re-immunoprecipitation (2° IP) with either preimmune rabbit serum (negative control), anti-lck and fyn kinase antisera, or with 4G10 anti-phosphotyrosine (anti-pY) mAb. The immunoprecipitates were washed and resolved by 10% SDS/PAGE.

GPI-anchored associated phosphoproteins has been described before and represents the classic pattern of GPI-anchored-protein associated phosphoproteins [1,13,36]. *In vitro* kinase assays performed on CD55 immunoprecipitates indicated the presence of the same pattern of phosphoproteins, though the 85 kDa band was lower in intensity and the 40 kDa band was nearly absent (on longer exposures the 40 kDa band could be observed). Curiously, *in vitro* kinase assays performed on CD109 immunoprecipitates revealed very few phosphoprotein associations. The 85 kDa phosphoprotein observed in the CD109 *in vitro* kinase assay was also present in the negative control and was not re-immunoprecipitated with an anti-phosphotyrosine mAb (see Figure 2, lower panel). Thus, this 85 kDa is both not specific for the GPI-anchored proteins and unlikely to be tyrosine-phosphorylated (it could be serine/threonine phosphorylated). The 85 kDa that is specifically associated with GPI-anchored proteins can be re-immunoprecipitated with an anti-phosphotyrosine mAb.

To gain a further understanding of the phosphoproteins co-immunoprecipitated with the GPI-anchored proteins, the products of the *in vitro* kinase assays were eluted off the immunoprecipitating Protein A–Sepharose beads in SDS and were subjected to re-immunoprecipitation with either anti-lck kinase rabbit antiserum, anti-fyn kinase rabbit antiserum, 4G10 anti-phosphotyrosine mAb or negative-control rabbit anti-serum, and were resolved using SDS/PAGE (Figure 2, lower panel). Anti-phosphotyrosine immunoprecipitation of the products labelled in the CD59 *in vitro* kinase assay demonstrated the presence of four major tyrosine-phosphorylated species: 120, 85, 50–60 and 40 kDa. Immunoprecipitation of the products labelled in the CD59 *in vitro* kinase assay with anti-lck and anti-fyn heterosera demonstrated that the 50–60 kDa tyrosine-phosphorylated species consisted of both lck and fyn tyrosine kinases. Similarly to the results obtained from the analysis of the CD59 *in vitro* kinase assay, anti-phosphotyrosine immunoprecipitation of the products labelled in the CD55 *in vitro* kinase assay demonstrated the presence of three major tyrosine-phosphorylated species (120, 85 and 50–60 kDa) and a fourth species (40 kDa) that could only be observed on overexposed autoradiographs. Also similarly to the analysis of the CD59 *in vitro* kinase assay, immunoprecipitation of the products labelled in the CD55 *in vitro* kinase assay with anti-lck and anti-fyn heterosera showed that the 50–60 kDa tyrosine-phosphorylated species consisted of both lck and fyn tyrosine kinases. Anti-phosphotyrosine immunoprecipitation of the products labelled in the CD109 *in vitro* kinase assay demonstrated that there was little or no tyrosine-phosphorylated protein co-immunoprecipitated with CD109. Not unexpectedly, lck and fyn heterosera also did not immunoprecipitate any observable material from the CD109 *in vitro* kinase assay.

Analysis of the phosphoproteins associated with GPI-anchored proteins from HSB cells (Figure 3) demonstrated that CD59 was associated with a pattern of tyrosine-phosphorylated proteins similar to that found in association with CD59 immunoprecipitated from HPB-ALL cells. Although, in contrast to the results from HPB-ALL cells, CD59 immunoprecipitated from HSB cells was only associated with lck src-family kinase, fyn kinase was absent from these immunoprecipitates. The absence of fyn in immunoprecipitates of GPI-anchored proteins expressed on HSB cells has been noted by us previously and is probably a reflection of the extremely high level of lck kinase expression in HSB cells [36]. In contrast to the results obtained for CD59, CD109 immunoprecipitates were only very weakly associated with tyrosine-phosphorylated proteins and with lck kinase.

Because the results obtained from T-cell tumour lines (HPB-ALL and HSB cells) indicated that there were substantial

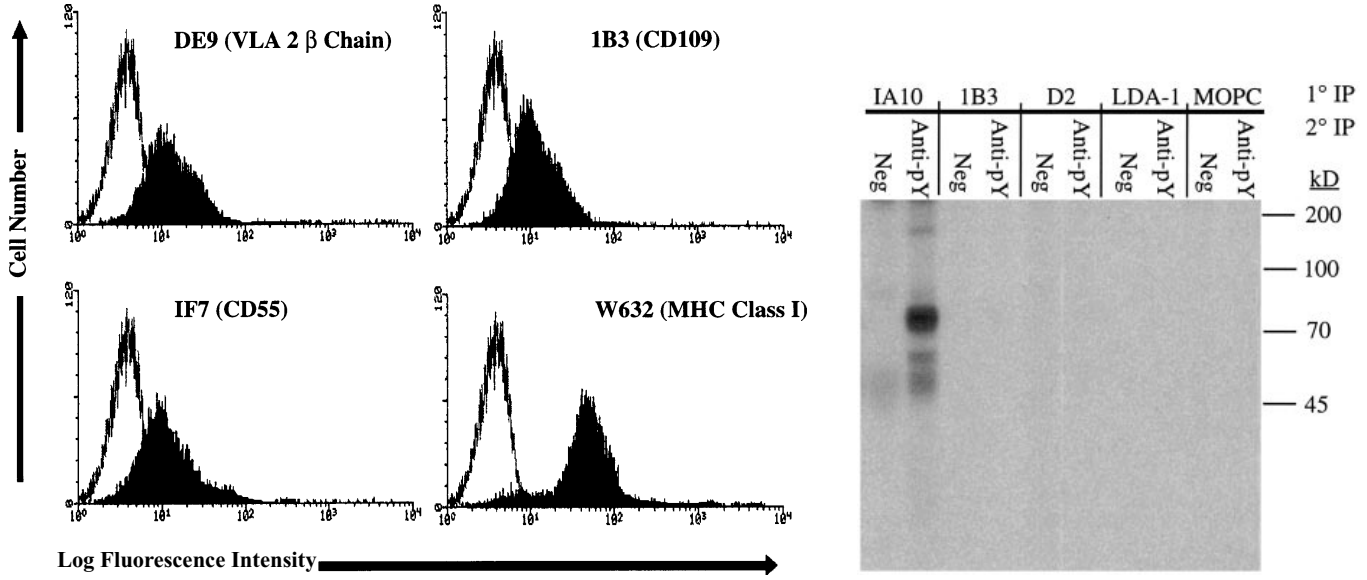

Figure 5 CD109 is not associated with kinases and phosphoproteins

(Left-hand panel) Immunofluorescence analysis of HPB-ALL integral membrane protein surface-expression levels. HPB-ALL cells were incubated with the indicated monoclonal antibodies followed by FITC-conjugate goat anti-mouse Ig. Data are plotted as cell number versus fluorescence intensity. (Right-hand panel) Absence of kinases and phosphoproteins from anti-CD109 immunoprecipitates. Lysates of HPB-ALL cells were subjected to immunoprecipitation (1° IP) with the indicated mAbs: IA10, 1B3, D2 and LDA-1 and MOPC (negative control), washed and subjected to *in vitro* kinase assays. The products of the *in vitro* kinase assays were eluted from the immunoprecipitating beads and subjected to re-immunoprecipitation (2° IP) with either preimmune rabbit serum (negative control), or with 4G10 anti-phosphotyrosine (anti-pY) mAb. The immunoprecipitates were washed and resolved by 10% SDS/PAGE.

differences in the ability of various GPI-anchored proteins to associate with phosphoproteins, we next examined the ability of GPI-anchored proteins to associate with phosphoproteins and kinases in non-transformed T cells (Figure 4). CD59, CD55 and CD109 were immunoprecipitated from PHA-stimulated T-cell blasts (CD109 is not expressed on resting T cells [35]) and *in vitro* kinase assay analysis was performed. Comparable with the results obtained from the T-cell tumour lines, CD59 and CD55 expressed on activated T cells were both associated with 85, 50–60 and 40 kDa tyrosine-phosphorylated proteins. There was no evidence in these immunoprecipitates of the 120 kDa tyrosine-phosphorylated protein co-immunoprecipitated with GPI-anchored proteins expressed on tumour cell lines. Immunoprecipitation of the *in vitro* kinase-labelled phosphoproteins with anti-lck and anti-fyn antisera revealed that CD59 and CD55 were associated with substantially more fyn kinase than lck kinase. Unlike the results obtained with CD55 and CD59, CD109 was associated with a very small amount of tyrosine-phosphorylated proteins. Thus, in two T-cell tumour lines and activated T cells, CD109 had very little association with kinases and other phosphoproteins.

The lack of phosphoproteins associated with CD109 was surprising, as nearly all other GPI-anchored proteins studied have been reported to associate with phosphoproteins and kinases [1,8–13,36]. Because this result was unusual we determined whether it was influenced by the level of CD109 expression or by the antibody with which we immunoprecipitated CD109. *In vitro* kinase assays were performed on CD55 and CD109 immunoprecipitates from lysates of HPB-ALL cells selected for equivalent expression of CD109 and CD55 (Figure 5, left-hand panel). Three different anti-CD109 mAbs (1B3, D2 and LDA-1) were used in these experiments to determine if the immunoprecipitating antibody was influencing our result (Figure 5, right-hand panel). Analysis of these *in vitro* kinase assays revealed that, whereas CD55 was associated with the tyrosine-phosphorylated proteins described above, CD109 (regardless of the immunoprecipitating antibody) was associated with little or no detectable phosphoprotein.

DISCUSSION

The non-ionic detergent insolubility of GPI-anchored proteins has been reported numerous times ([14–20] and many others) and cited with even greater frequency, yet few studies actually measuring this insolubility have been performed. Moreover, whereas various properties associated with GPI-anchored proteins, i.e. detergent insolubility and signal-transduction molecule association, have been hypothesized to originate from the inclusion of GPI-anchored proteins in lipid microdomains containing high concentrations of cholesterol and sphingolipids, the relationship between these properties has not been established. Studies measuring solubility have been performed on non-haemopoietic cells, whereas studies detecting kinase/heterotrimeric G protein associations and signal transduction have been performed on haemopoietic cell types. In this report we demonstrated that the Triton X-100 insolubility of GPI-anchored proteins expressed on T-cell tumours and activated T cells varies considerably. We also demonstrated that the presence of kinases and phosphoproteins in association with GPI-anchored proteins also varies dramatically. The variation in detergent insolubility correlates with the kinase/phosphoprotein association variation, thus supporting the hypothesis that these two properties are related.

In these studies we demonstrated that there is considerable variation in the non-ionic detergent solubility of various GPI-anchored proteins: CD59 was between 4% and 13% soluble, CD48 was between 13% and 25% soluble, CD55 was between 20% and 30% soluble, and CD109 was between 34% and 75%

soluble. The solubility of these three molecules always followed the pattern CD109 > CD55 > CD48 > CD59 in all cell lines tested. This type of variation in the non-ionic detergent insolubility of GPI-anchored proteins has been observed previously for GPI-anchored proteins expressed on kidney microvillar membranes [14]. In those studies the range of Triton X-100 solubilities ranged from approx. 15 % to 60 % (for four different GPI-anchored proteins), which is close to the range of solubilities measured in this study. The solubility levels of the proteins measured here are only for monomeric, completely solubilized, forms of the studied proteins. In some experiments, either incompletely solubilized forms of the GPI-anchored proteins (in higher-molecular-mass complexes with other proteins and lipids), or other proteins associated with the GPI-anchored proteins [37], were present in our gels. These other species were not included in our solubility analysis as our goal was to measure the solubilization of the GPI-anchored proteins.

It is interesting to speculate on the origin of the variation in the non-ionic detergent solubility of GPI-anchored proteins. The variation in solubility appeared to correlate with the molecular mass of the protein: CD109 is 120–180 kDa, CD55 is 70 kDa, CD48 is 43 kDa, whereas CD59 is 18–20 kDa. This interpretation is consistent with studies in which lipid-anchored (either lipopeptide or phospholipid moieties) poly(ethylene glycol) (PEG) conjugates of various molecular masses were measured for spontaneous intervesicle transfer. Those studies revealed that the rate of transfer correlates with the size of the PEG group. Thus, the larger the PEG conjugate the faster the spontaneous transfer rate [38]. Since it is likely that the spontaneous intervesicle transfer rate measured in those studies is related to detergent solubility, it is also likely that it can be assumed generally that the molecular mass of the groups attached to lipid anchors has an influence on detergent solubility. Since we demonstrated a correlation between the molecular mass of the GPI-anchored proteins and their detergent solubility, it is possible that the observations made in those previous studies also apply to the results described here.

A second potential cause of the variation in the non-ionic detergent insolubility of the different GPI-anchored proteins is in the percentage of a given protein sorted to the detergent-insoluble microdomains during Golgi transport. It is possible that for some GPI-anchored proteins only a fraction of the total molecules are found in the microdomain lipid self-assemblages. For instance, only 50 % of CD109 may be sorted to the detergent-insoluble lipid microdomain, whereas the other 50 % is found in the general membrane-lipid milieu.

Another possible origin for the different solubilities could be due to variation in the alkyl/acyl groups attached to the GPI-anchor. The presence of an acylated inositol ring would have an impact on the detergent solubility of GPI-anchored proteins. The presence of this anchor modification would also be detected in the degree to which these molecules were cleavable by phosphatidylinositol-specific phospholipase C (PI-PLC). Since the non-PI-PLC-cleavable fraction of each of these molecules was between 1 % and 10 % (results not shown) this would appear to be an unlikely origin for the variation in detergent solubility. Studies performed in lipid vesicles by Peitzsch and McLaughlin [39] demonstrated that the energy of the interaction of acylated peptides with phospholipid vesicles increases by 0.8 kcal/mol for every additional carbon in the fatty acid. Therefore, myristic acid has 12 kcal/mol of membrane-binding energy, whereas stearic acid has approx. 15.2 kcal/mol of membrane-binding energy. To emphasize the impact of this difference on membrane association the authors point out that only 40 % of a protein with an acyl chain of 12 carbons would be bound to the membrane, whereas 99 % of a protein with a 16 carbon acyl chain would be membrane-bound [39]. Thus, variation (including the degree of saturation) in the fatty acid portion of the GPI anchors could account for the observed differences in detergent solubilities. Such an explanation would require that different GPI anchors would be attached to different GPI-anchored proteins within the same cell. Protein-specific GPI anchors within *Saccharomyces cerevisiae* have been described [40], suggesting that protein-specific GPI-anchor variation may also exist in mammalian cells. Since there was a surprising degree of consistency between the GPI-anchored protein solubilities measured from the three cell types tested, a necessary condition for this model is that the mechanism by which the anchor variation arises is conserved. Interestingly, we found that the src kinases, which are dual acylated and which were found in association with GPI-anchored proteins (lck/fyn), were approx. 70 % resistant to extraction with Triton X-100, whereas pp60 src, which is not lipid modified and is not found in association with GPI-anchored proteins [41], is > 90 % soluble in Triton X-100 (results not shown). Thus, it appears that the presence and types of lipid modification found on proteins have a profound influence on their detergent solubility.

It has been demonstrated that > 50 % of membrane lipids are resistant to Triton X-100 extraction [42] and, thus, sphingolipid/cholesterol-rich membrane microdomains are not the only Triton X-100-insoluble membrane fraction. It is likely that non-ionic detergent-insoluble membrane domains are also present in Golgi and endocytic compartments. Other detergent-insoluble fractions may be found in other membranes, and various types of detergent-insoluble fraction may be present on the cell surface. Proteins may be resistant to Triton X-100 extraction for a variety of reasons. High concentrations of sphingolipids and cholesterol result in the Triton X-100 insolubility [22], as does the interaction with the cytoskeleton, and protein-specific modifications (such as lipid anchors) may also contribute to non-ionic detergent insolubility. There may also be other reasons for Triton X-100 insolubility that have not been described. Thus, Triton X-100 insolubility alone is not a criterion for assuming that a protein is in a membrane microdomain. In the studies performed here we were able to show that whereas the GPI-anchored proteins studied resisted Triton X-100 extraction, they were well-solublized by Triton X-100 plus octylglucoside. Therefore, the detergent insolubility of the proteins was not due to cytoskeletal association. The solubility studies performed here were on iodinated proteins, which indicated that they were cell-surface derived. Unlike other techniques, i.e. immunoblotting, our methodology eliminated any contribution of non-cell-surface membranes from our analysis. Due to the fact that the GPI-anchored proteins studied here were solubilized by octylglucoside and were cell-surface derived, it is reasonable to speculate that the Triton X-100 insolubility of the GPI-anchored proteins observed in these studies may at least partially derive from the inclusion of the proteins in cell-surface-membrane microdomains.

The ability of GPI-anchored proteins to associate with phosphoproteins varied considerably. CD59 was associated with multiple tyrosine-phosphorylated proteins including lck and fyn kinases and a 40 kDa phosphoprotein species that we had identified previously as multiple α subunits of heterotrimeric G proteins [13]. CD48 was associated with a similar pattern of phosphoproteins, while in CD55 immunoprecipitates the 40 kDa heterotrimeric G protein α subunit was difficult to detect. In contrast, CD109 had very low levels of associated phosphoproteins. It could be argued that the inability to detect phosphoproteins associated with CD109 was not due to the

absence of many of the phosphoproteins but only due to the lack of kinases. Though this possibility was remote, we tested whether CD109 co-immunoprecipitated with CD55 would alter the amount or pattern of phosphoproteins associated with either of the molecules (results not shown). *In vitro* kinase assays performed on CD55 immunoprecipitates with or without CD109 co-immunoprecipitation were identical. Thus, CD109 immunoprecipitates did not contribute any proteins that could be phosphorylated in the *in vitro* kinase assay (nor did CD109 co-immunoprecipitation alter the kinase activity associated with CD55) despite the abundant kinases associated with CD55.

The pattern of phosphoprotein association and the variation between the different GPI-anchored proteins was similar from cell type to cell type, with some notable exceptions. In HSB cells GPI-anchored proteins were associated much more with lck kinase than with fyn kinase. This is in contrast to kinases associated with GPI-anchored proteins expressed on either HPB-ALL cells or PHA-activated T cells, which have more fyn than lck kinase. This difference has been noted by us previously [36] and is probably the result of the chromosomal translocations that cause much higher levels of lck expression in HSB cells [43,44]. Another difference in the pattern of phosphoproteins was found in the comparison of the phosphoproteins associated with GPI-anchored proteins in T-cell tumour lines with those associated with GPI-anchored proteins in T-cell blasts. In PHA-activated T cells the 115–120 kDa phosphoprotein observed in the *in vitro* kinase assays of GPI-anchored proteins expressed on either HPB-ALL cells or HSB cells was not apparent. The identity of this phosphoprotein is unknown and the reason for its absence is unclear.

Interestingly, the amount of phosphoprotein associated with a particular GPI-anchored protein varied inversely with its Triton X-100 solubility. Thus, the more insoluble the GPI-anchored protein was, the more it was associated with kinases and other phosphoproteins. It is therefore necessary to conclude that two types of solubilities were measured in these studies. The first is the separation of the GPI-anchored protein with its associated lipids from the general lipid membrane, and the second is the complete solubilization of the GPI-anchored protein away from its associated lipid microdomain. The former accounts for the low levels of detergent solubility found for CD59, and the latter for the high levels of detergent solubility found for CD109.

The type of analysis performed in this report revealed substantial differences in the ability of GPI-anchored proteins to associate with kinases and phosphoproteins, but did it reveal anything about the function of these proteins? Both CD55 and CD59 are regulators of complement function. CD55 (DAF) functions by preventing the assembly of the C3 and C5 convertases [45], whereas CD59 binds to the C8 and C9 components of complement and prevents formation of the membrane-attack complex [46,47]. CD59 may also bind to CD2, a T-cell/natural-killer cell antigen, with low affinity [48]. Human CD48 is also a low-affinity ligand for CD2 [49], whereas CD109 has no known function (K. R. Solomon and R. W. Finberg, personal observation). Although it has not been demonstrated that physiological ligands (complement, CD2) can induce signalling from CD55, CD59 or CD48, all three receptors have all been shown to induce signal transduction, including tyrosine phosphorylation, when cross-linked with mAbs [1–3,5,6,10]. This suggests a role for non-receptor tyrosine kinases in signal propagation from these GPI-anchored proteins. It is worth noting that we have been unable to demonstrate any signal transduction induced by the cross-linking of CD109 despite attempting these studies with seven different mAbs used in various combinations (K. R. Solomon and R. W. Finberg, personal observation). It would be premature to conclude that CD109 cannot signal based on these observations due to the fact that not all mAbs raised to CD55 or CD59 can induce signal transduction when cross-linked (though all the ones we have tested maintain the protein/phosphoprotein associations in *in vitro* kinase assays) ([5] and K. R. Solomon and R. W. Finberg, unpublished work). It is tempting to speculate that the low levels of phosphoproteins associated with CD109 are related to our inability to generate signals by cross-linking CD109, but this analysis should be viewed with caution as we did not attempt to measure all types of signals that may be generated from a cross-linked receptor (we measured proliferation, growth inhibition, calcium mobilization and tyrosine phosphorylation).

The studies presented here quantificate for the first time the non-ionic detergent insolubility of various GPI-anchored proteins expressed on T cells. These studies revealed a positive correlation between Triton X-100 solubility and molecular mass and an inverse correlation with kinase activity and phosphoprotein association. The link between insolubility and phosphoprotein association supports a model of GPI-anchored protein/cytoplasmic-messenger association via co-localization to detergent-insoluble lipid microdomains.

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