Recombinant glycosyl-phosphatidylinositol-anchored proteins are not associated with protein kinases in transfected thymoma cells

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The cross-linking by antibody of some glycosyl-phosphatidylinositol (GPI)-anchored proteins on the plasma membrane of T cells leads to cell activation. Phosphorylation of proteins on tyrosine residues has a central role in the control of T cell activation, and non-receptor protein tyrosine kinases can be co-precipitated with immune complexes of GPI-anchored proteins in T cell lysates. In order to investigate the nature of this interaction, two recombinant GPI-anchored proteins were constructed (using the GPI signal sequence from Thy-1), and their associations with protein tyrosine kinases in stable transfectants of a mouse thymoma have been investigated. One recombinant GPI protein is the extracellular domain of the human complement receptor-1, normally an integral membrane protein, and the

other is the secreted protein, human tissue inhibitor of metalloproteinases. The latter protein should be foreign to the cell surface and yet has been expressed as a GPI-anchored protein at levels equivalent to the highly expressed antigens Thy-1 and Ly6.A2 on mouse thymoma cells. Neither of the two recombinant proteins, when immunoprecipitated from NP40 lysates of transfected cells, was associated with protein tyrosine kinases in contrast with the natural endogenous GPI-anchored proteins Thy-1 and Ly6.A2 in non-transfected parental cells. Moreover, high expression of foreign recombinant GPI protein appears to interfere with the association of the natural GPI proteins with protein tyrosine kinases.

INTRODUCTION

Surface membrane proteins of many diverse families can be anchored to the cell membrane through a glycosyl-phosphatidylinositol (GPI) structure (reviewed in Ferguson and Williams, 1988). These proteins do not penetrate the lipid bilayer via a transmembrane hydrophobic domain, or have a cytoplasmic domain, as do integral transmembrane proteins. Instead, the acyl/alkyl or diacylglycerol carbon side chains attached via phosphate to the inositol group anchor the protein in the outer lipid bilayer by hydrophobic interaction. Although this method of linkage does not seem to allow any contact between the GPI-anchored protein and proteins located at the inner cytoplasmic face of the cell membrane, GPI-anchored proteins are often associated with non-receptor protein kinases of the *src* family in NP40 detergent extracts (Stefanova et al., 1991; Thomas and Samelson, 1992). These kinases autophosphorylate and phosphorylate other proteins on tyrosine, forming part of the signalling cascade between receptors on the cell's exterior, and regulation of structural changes to the cytoskeleton and nuclear transcription (reviewed for lymphocytes in Samelson and Klausner, 1992; Mustelin and Burn, 1993).

GPI-linked proteins are not efficiently solubilized in NP40 cell lysates (Hooper and Turner, 1988) and can be isolated in a discrete lipid-rich fraction by centrifugation on sucrose density gradients (Sargiacomo et al., 1993), or sedimented at 200000 *g* (Draberova and Draber, 1993). In such fractions the GPI-linked proteins are in large complexes of about 100 nm diameter (Cinek and Horejsi, 1992) which are thought to correspond to glycosphingolipid-rich microdomains in the cell membrane (Brown and Rose, 1992). These complexes, when isolated from many different haemopoietic cell types, contain protein tyrosine kinases in addition to several GPI-anchored membrane proteins (Cinek and Horejsi, 1992; Draberova and Draber, 1993). Octyl glucoside

disrupts this association (Thomas and Samelson, 1992) and also disperses the Triton X-100/NP40 insoluble complexes (Sargiacomo et al., 1993) containing the GPI-linked proteins and protein tyrosine kinases. Other groups have found that the large complexes isolated from MDCK cells (Sargiacomo et al., 1993) and from Fischer rat thyroid (FRT) cells (Zurzolo et al., 1994) contain the protein caveolin and that they are absent from a mutant FRT cell line unable to synthesize caveolin protein (Sargiacomo et al., 1993; Zurzolo et al., 1994). These data suggest that in epithelial cells the glycosphingolipid-rich microdomains correspond to the caveolae (Ying et al., 1992) which appear as invaginations in electron micrographs on the cell surface of many different epithelial cell types.

Presumably it is the physical nature of the lipid moiety of GPI-anchored proteins which directs these proteins to lipid microdomains. Is the association with *src*-related kinases a result of their common occupancy of glycosphingolipid-rich domains or does it result from a more specific interaction? To answer this question I have transfected mouse thymoma cells with recombinant GPI-linked proteins. The last 37 amino acids of the GPI-linked protein, human Thy-1, were placed at the end of a secreted protein, tissue inhibitor of metalloproteinases-1 (TIMP) (Clissold, 1992), and also used to replace the transmembrane and cytoplasmic domains of the Complement Receptor-1 (CR1) (Clissold et al., 1993) by genetic engineering. The Thy-1 sequence constitutes a consensus signal sequence for GPI anchorage and during intracellular maturation and processing of the chimaeric protein all but six amino acids of Thy-1 are removed and replaced by the GPI structure. It has been previously shown that these chimaeric proteins are expressed on the transfected cells' surface and can be cleaved off by phosphoinositol-specific phospholipase C (Clissold, 1992; Clissold et al., 1993) and labelled by [¹⁴C]ethanolamine, a constituent of the anchor, showing the proteins to be GPI-anchored. I have looked for their

Abbreviations used: GPI, glycosyl-phosphatidylinositol; FRT, Fischer rat thyroid; TIMP, tissue inhibitor of metalloproteinases-1; CR-1, complement receptor-1; ECL, enhanced chemiluminescence.

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association with protein tyrosine kinases in both NP40 and octyl glucoside lysates from the transfected thymoma cells. The loss of association of naturally linked GPI-proteins when engineered to become integral transmembrane proteins with the kinases has been documented (Shenoy-Scaria et al., 1992). To my knowledge, however, the converse situation of placing proteins which are not normally GPI-linked on such an anchorage and then looking for interactions with protein tyrosine kinases has not been investigated. If a specific bridging protein is required for the interactions which bind the GPI-linked protein to the cell exterior and the kinase to the interior, then a 'foreign' recombinant GPI protein may not be able to participate in the binding. Alternatively, if they co-reside with the kinases solely because their lipid tails ensure that they go to the same microdomains, then a 'foreign' exterior domain should not affect the association. In this paper I report on the results of experiments designed to test these ideas.

METHODS

Materials

Monoclonal antibody to Thy-1 (rat IgG2b, clone number 154.7), was a gift from Steve Cobbold (Cobbold et al., 1989). Antibodies to mouse H2K^k (mouse IgG2a, clone 36-7.5) and CD11a of LFA-1 (rat IgG2b, clone 2D7) were purchased from Pharmingen, San Diego, CA, U.S.A. Antibody to Ly-6.A2 (mouse IgG2, clone 5041-24.2) was from Serotec (Oxford, U.K.) and control MOPC 21 IgG1 was from Sigma. Antibody to TIMP, clone RRU-T1, mouse IgG1 was a gift from Ian Clarke and Tim Cawston. The CR1 antibody, mouse IgG1 clone 4D2 was a gift from SmithKline and Beecham. Monoclonal anti-phosphotyrosine conjugated to agarose, clone PY-20, was bought from Transduction Laboratories, Lexington, KY, U.S.A. Protein G agarose beads were from Pierce and anti-mouse IgG1 (H-chain specific) agarose beads were from Sigma. The monoclonal antibody to CD45 (rat IgG2b) was also a gift from S. Cobbold. ¹⁴C-labelled Rainbow[™] protein molecular weight markers and [γ -³²P]ATP were from Amersham International. Protease inhibitors were from Boehringer, sodium orthovanadate from LC Laboratories (Woburn, MA, U.S.A.) and all other chemicals were from Sigma. Cell culture reagents were also purchased from Sigma. Phosphoinositol-specific phospholipase C from *Bacillus thuringiensis* was purchased from Oxford Glycosystems.

Cell lines

The cell lines used in these experiments were the parental non-transfected cell line BW 5147, a mouse thymoma, and stable transfected BW 5147 cell lines expressing either GPI-TIMP or GPI-CR1. The construction of these plasmids, cell transfections and assay of expression of the chimaeric GPI-anchored proteins have been described previously (Clissold, 1992; Clissold et al., 1993). Cells were maintained in Dulbecco's modified Eagle's medium and 10% foetal calf serum. Transfected cell stocks were maintained under selection in 2.5 mg/ml Geneticin (GIBCO/BRL) but were grown for 48 h in its absence prior to lysis and immune precipitation.

Surface antigen expression levels were analysed by fluorescence flow cytometry using primary antibodies followed by fluorescein-conjugated secondary antibodies on living cells with a Becton-Dickinson FACScan and the Lysis-2 programme. Secondary antibodies (either anti-mouse IgG or anti-rat IgG) conjugated to fluorescein, were purchased from Sigma.

Antibody selection of surface antigens

Cells were lysed at a concentration of 5×10^7 /ml in 50 mM

Tris/HCl/0.15 M NaCl/2 mM EDTA, pH 8.0/1% NP40 (lysis buffer) for 40 min on ice. In experiments with octyl glucoside a concentration of 60 mM (Hooper and Turner, 1988) was used instead of the NP40. Sodium *o*-vanadate (1 mM) was present at all times to inhibit phosphatases with the protease inhibitors leupeptin at 10 μ M, pepstatin at 3 μ M, phosphoramidon at 20 μ M and phenylmethylsulphonyl fluoride at 100 μ M. After 40 min of incubation on ice, cell lysates were centrifuged at 14000 rev./min in an Eppendorf microfuge in the cold-room for 10 min. Supernatants were removed and divided into 200 μ l aliquots each equivalent to 1×10^7 cells. Lysates were pre-cleared with an equal mix of Protein G and anti-IgG1 beads for 2 h at 4 °C.

Protein G beads or anti-mouse IgG1 beads (for mouse IgG1 isotypes) were preincubated with 20 μ g of the monoclonal antibodies in 400 μ l of 0.15 M NaCl/25 mM Hepes, pH 7.2/0.1% NP40 (wash-buffer) for 24 h at 4 °C. (In experiments using octyl glucoside lysis, 6 mM octyl glucoside replaced 0.1% NP40 in the wash buffer.) Beads were washed in this buffer with the addition of 2 mM EDTA and 10 μ l of beads (having bound a possible maximum of 6.6 μ g of monoclonal antibody) and were incubated with lysate equivalent to 1×10^7 cells (i.e. 200 μ l lysate) at 4 °C for 4 h. After binding of cellular antigens, unbound proteins were removed in the supernatants from the beads which were then washed twice in wash buffer and then once in kinase buffer, 1 ml each wash. In preliminary experiments the amount of antibody and protein G beads were shown to be in excess, as re-using the antibody-protein G beads on a fresh cell lysate did not change the results obtained in the kinase reactions (see next section).

Kinase reactions

Kinase reactions were performed on the immune complexes on the beads in 25 μ l/sample of 5 mM MnCl₂/5 mM MgCl₂/50 mM Hepes, pH 7.2/10 μ Ci of [γ -³²P]ATP (3000 Ci/mmol) for 5 min at room temperature. Beads were then washed in wash buffer without detergent but with 2 mM EDTA. SDS (20 μ l; 2%) was added to the beads and they were boiled for 2 min to release the immune complexes from the beads. Then a 500 μ l volume of 1% NP40 lysis buffer containing all inhibitors was added to each sample and all supernatant was removed from the Protein G or anti-IgG1 beads. Supernatants were transferred to 20 μ l of anti-phosphotyrosine agarose beads to select for proteins having phosphorylated tyrosine residues. Selection was for 14 h at 4 °C. At the end of this time the supernatants were removed from the anti-phosphotyrosine beads which were washed once in EDTA-containing wash buffer with 0.1% NP40, and then twice without detergent. Sodium *o*-vanadate at 200 μ M was present in all washes. Beads were then boiled in Laemmli reducing sample buffer to release bound proteins and these were separated according to size by electrophoresis through a 9% Laemmli polyacrylamide gel (Laemmli, 1970). After electrophoresis the gel was fixed for 30 min in 20% methanol/10% acetic acid, dried down and autoradiographed at -70 °C with intensifying screens.

Western blotting and enhanced chemiluminescence (ECL) detection of antigens

Proteins separated by electrophoresis through SDS/polyacrylamide gels were Western blotted to Millipore Immobilon P sheets using a Hoefer semi-dry blotter according to the manufacturer's instructions. Towbin's buffer (Towbin et al., 1979) containing 10% methanol was the transfer buffer. Blocking buffer was PBS containing 0.1% Tween 20 and 0.5% gelatine.

Primary antibodies were at 3 $\mu\text{g}/\text{ml}$ (RRU-T1 and 4D2 as above) and biotinylated secondary antibodies were bought from Amersham. The latter were ECL grade and were used as in the manufacturer's instructions. The ECL detection reagents were also from Amersham and used as in their directions. Biotinylated protein markers (Broad Range) were from Bio-Rad.

RESULTS

The association of protein tyrosine kinases with four cell surface antigens, two of which were transmembrane (H2K^k and LFA-1) and two naturally GPI-anchored (Thy-1 and Ly6.A2), was compared with that of two proteins engineered to be GPI-linked (human TIMP and CR1). Natural TIMP is a secreted protein and has been engineered to be a cell surface protein by placing it on a GPI-anchor. CR1 is normally a transmembrane protein expressed on the surface of haemopoietic cells. In both cases GPI-anchorage was conferred by using the GPI signal sequence of Thy-1 (Clissold, 1992; Clissold et al., 1993).

Relative levels of surface antigens of parental and transfected BW 5147 cells

Flow cytometry analysis showed that cells transfected with GPI-TIMP or GPI-CR1 expressed the same levels of Thy-1 antigen as the non-transfected parental cells (Figure 1). Neither was there

Figure 1 Flow cytometry analysis of the cell lines labelled with fluorescent antibody

(a) Parental non-transfected BW 5147 thymoma cells; (b) BW 5147 cells transfected with GPI-TIMP from FACS sort 2, clone 6; (c) BW 5147 cells transfected with GPI-CR1 from a twice FACS-sorted population, clone 19-B6. Antibodies: in each case the continuous line at the left-hand side is MOPC 21, IgG1 isotype, which is the negative control. The numbers denote antibodies to the following cell surface markers: (1) H2K^k, (2) LFA-1, (3) Thy-1, (4) Ly6.A2, (5) TIMP (6) CR1. The x-axis is the logarithmic scale of fluorescence intensity (to 10^4) and the y-axis is the relative cell number.

any change in the level of expression of the two transmembrane proteins H2K^k or LFA-1 antigens between cell lines. The level of Ly6.A2 expression, however, was increased 5-fold in cells transfected with GPI-TIMP (Figure 1b, trace 4). The amount of expression of GPI-TIMP (Figure 1b, trace 5) in cells transfected with the GPI-TIMP plasmid, was of the same order of magnitude as that of Thy-1 (trace 3) on all cells, but that of GPI-CR1 on stable transfected GPI-CR1 cells was 50-fold lower (Figure 1c, trace 6). All of the cell clones and the parental non-transfected cells expressed the same high levels of the receptor tyrosine phosphatase CD45 (results not shown).

The association of protein kinases with natural and recombinant GPI-linked proteins

The molecular masses of ^{32}P -labelled proteins in immune complexes on beads after incubation with ^{32}P but before selection by antibody to phosphotyrosine were investigated in preliminary experiments. Autoradiography of dried polyacrylamide gels showed many phosphorylated proteins but there were no specific differences between immune complexes precipitated by the six different antibodies or by the control MOPC 21 antibody (results not shown). Presumably many of the bands were due to serine/threonine kinases or other non-physiological phosphorylations brought about by disruption of the cellular architecture.

In all following experiments, therefore, phosphotyrosine-containing proteins were specifically selected. After initial antibody selection of the cell surface antigens and *in vitro* phosphorylation of any associated kinases and their protein substrates, the immune complexes were disrupted by SDS treatment. Subsequently, under re-naturing conditions (1% NP40), the tyrosine-phosphorylated proteins which had been immuno-co-selected, were then directly immunoprecipitated with a phosphotyrosine monoclonal antibody conjugated to agarose beads. Results from using this second round of selection were consistent in showing a clear difference in the association of different cell surface antigens with tyrosine kinase activity and tyrosine phosphorylated proteins.

In NP40 lysates from all three cell types, neither H2K^k nor LFA-1 was associated with protein tyrosine kinases capable of autophosphorylation or phosphorylation of other proteins in the *in vitro* kinase reaction on immune complexes (Figures 2a, 2b and 2c, lanes 1 and 2). The naturally GPI-anchored proteins Thy-1 and Ly6.A2 were associated, however, with proteins which were phosphorylated on tyrosine residues *in vitro* (Figure 2a, lanes 3 and 4). They co-precipitated with a very prominently phosphorylated protein migrating on PAGE at a position corresponding to an M_r of 90000 which was not present in immune complex precipitations of negative controls (i.e. the absent antigens GPI-TIMP and GPI-CR1 on the non-transfected parental cell line) or in MOPC 21 controls in any of the cell lines. In kinased immune complexes from non-transfected cells the irrelevant control antibody MOPC 21 track (Figure 2a, lane 7) looked the same as the tracks from immune complexes from the absent antigens GPI-TIMP and GPI-CR1 (Figure 2a, lanes 5 and 6). Moreover the tracks from these antigen selections looked the same in cells transfected with GPI-TIMP (Figure 2b, lanes 5, 6 and 7) and in cells transfected with GPI-CR1 (Figure 2c, lanes 5, 6 and 7). The bands seen corresponded to the positions of major bands in the total lysate track (lane T) and in the MOPC 21 tracks (lanes 7), and so seemed to be non-specific and due to selection using anti-mouse IgG1 agarose beads rather than Protein G beads. In view of the high backgrounds when using anti-IgG1 beads, in later experiments Protein G beads were used for collection of anti-mouse IgG1 immune complexes. Western blots of proteins bound during the immune complex formation

Figure 2 Autoradiograph of phosphotyrosine proteins ^{32}P -labelled *in vitro* in immune complexes from NP40 cell lysates separated by SDS/PAGE

(a) Parental non-transfected BW 5147 thymoma cells; (b) BW 5147 cells transfected with GPI-TIMP from FACS sort 2, clone 6; (c) BW 5147 cells transfected with GPI-CR1 from a twice FACS-sorted population, clone 19-B6. Precipitating antibodies: (1) H2K^b, (2) LFA-1, (3) Thy-1, (4) Ly6.A2, (5) TIMP, (6) CR1, (7) MOPC 21. After capture by the above antibodies pre-bound to either protein G or anti-mouse IgG1 agarose beads (see the Methods section) cell proteins were subjected to *in vitro* kinasing. After release from the immune complexes by SDS, proteins were renatured in NP40. Co-immunoprecipitated phosphotyrosine proteins were then recaptured with the anti-phosphotyrosine monoclonal antibody PY20 attached to agarose beads, and then separated on 9% SDS/PAGE. The total lane (T) is unselected cell lysate from non-transfected cells. The marker lane (M) is Amersham's ^{14}C -Rainbow Markers.

Figure 3 A Western blot from a 5–15% gradient SDS/PAGE of immune complexes selected by TIMP monoclonal antibody and protein G beads

The detection system was Amersham's ECL using streptavidin conjugated horseradish peroxidase and a phosphorescent substrate. The primary antibody was to TIMP and the second antibody was biotinylated anti-mouse IgG1 from Amersham. Exposure was for 5 s. Markers (biotinylated broad range protein markers from BioRad) are on the left. Lane 1, parental non-transfected BW 5147 thymoma cells. Lane 2, BW 5147 cells transfected with GPI-TIMP, clone 2.6. Lane 3, BW 5147 cells transfected with GPI-CR1, clone 19-B6. The arrow denotes the GPI-TIMP of M_r 30 000 in lane 2. (The bands in all tracks of M_r 25 000 are the precipitating antibody from the immune complexes which is recognized by the second biotinylated antibody used in the ECL detection system.)

of cell surface antigens showed that GPI-TIMP had been selected by the immune precipitation procedure with Protein G beads from the GPI-TIMP transfectants (Figure 3, lane 2). In these experiments there was less background and still no specific association of the recombinant GPI-linked proteins with kinases compared with the natural GPI protein, Thy-1 (Figure 6).

The phosphorylated protein of M_r 90 000 was specific to Thy-1 and Ly6.A2 antibody-selected immune complexes. It was very high in parental non-transfected cells (Figure 2a, lanes 3 and 4) but was nearly absent from Thy-1 and much lower in Ly6.A2

Figure 4 Octyl glucoside lysis

Autoradiograph of phosphotyrosine proteins ^{32}P -labelled *in vitro* in immune complexes from octyl glucoside lysates separated by 9% SDS/PAGE. N.B. Lane 4 is an anti-Thy-1-precipitated immune complex from an NP40 lysate of non-transfected cells (from the same flask as the octyl glucoside lysates) kinased and run in parallel with the octyl glucoside samples. Sets of cell lysates (a), (b) and (c) as for Figure 2. Precipitating antibodies: (a) (1) H2K^b, (2) LFA-1, (3) Thy-1, (4) Thy-1 in 1% NP40, (5) Ly6.A2, (6) TIMP, (7) CR1, (8) MOPC 21. (b) (1) H2K^b, (2) LFA-1, (3) Thy-1, (4) Ly6.A2, (5) TIMP, (6) CR1, (7) MOPC 21. (c) (1) H2K^b, (2) LFA-1, (3) Thy-1, (4) Ly6.A2, (5) TIMP, (6) CR1. All lanes are from a 60 mM octyl glucoside lysate with the exception of (a, lane 4), which is from a 1% NP40 lysate. The total (T) lane is from an octyl glucoside lysate of non-transfected cells selected only with anti-phosphotyrosine beads after *in vitro* kinasing. (The lysate was diluted 10-fold in the kinase buffer.) Markers (M) are from Amersham as in Figure 2.

antibody-selected immune complexes from GPI-TIMP transfectants (Figure 2b, lanes 3 and 4). Its association with Thy-1 was also much weaker in GPI-CR1 transfectants (Figure 2c, lane 3). These results were surprising as Thy-1 was expressed to an equal extent on the surface of all three cell lines. Ly6.A2 levels were comparable between parental cells and GPI-CR1 transfectants but was even more highly expressed (5-fold) on the surface of GPI-TIMP transfectants of clone 2.6 (Figure 1b, trace 4).

Octyl glucoside lysis

When the whole experiment was repeated using 60 mM octyl glucoside for lysing the cells, the proteins selected by phosphotyrosine antibody, which were specific to Thy-1 and Ly6.A2 immune complexes, decreased drastically as denoted by their *in vitro* phosphorylation (Figure 4). The protein with an M_r of 90 000 co-selected with Thy-1 was far less prominent in octyl glucoside lysates of parental cells (Figure 4a, lane 3) than in a 1% NP40 lysate (Figure 4a, lane 4). These two samples came from the same batch of cells, lysed on the same day, kinased with the same batch of kinase mix, and all procedures run in parallel.

The association of protein tyrosine kinases with GPI proteins in different cell clones of transfectants

Since the transfectants had all been cloned the differences detailed above may have been due to clonal variation. Immune co-precipitations in 1% NP40 lysates were repeated on four different clones of GPI-TIMP transfectants, one of which was resistant to Geneticin but had lost its expression of GPI-TIMP

Figure 5 Flow cytometry analysis of the cell surface expression of GPI-linked antigens on five different clones of GPI-TIMP transfected BW 5147 cells and non-transfected parental cells

The x- and y-axes are the same as in Figure 1. The hatched area is non-transfected cells with second fluorescein isothiocyanate-conjugated antibody alone. (a) GPI-TIMP, (b) Ly6.A2, (c) Thy-1. Symbols: —, non-transfected parental cells; ×—×—×, GPI-TIMP transfected cells, sub-clones 2.5, 2.6 and 2.7; ○—○—○, GPI-TIMP transfected cells, sub-clone 2.9; ----, GPI-TIMP transfected cells, sub-clone 2.8.

(see the flow cytometry results for clone 2.8 in Figure 5). All of these clones were derived from a FACS sort for high expressors followed immediately by cell cloning. The surface antigens investigated with respect to their association with protein tyrosine kinases were Thy-1, Ly6.A2 and GPI-TIMP using Protein G beads to select the immune complexes (and not anti-IgG1 for the TIMP immune complex as before). Results (Figure 6) showed that Thy-1 was uncoupled from protein tyrosine kinases in cells from clones 5 and 7 (FACS sort 2) expressing very high levels of GPI-TIMP (flow cytometry results in Figure 5) but not in the cells from clone 2.8 which had lost all GPI-TIMP expression (Figure 5a). In the progeny from clone 2.9, which had half the amount of GPI-TIMP compared with that of cells from clones 2.5, 2.6 and 2.7 (Figure 5a), there was an intermediate level of *in vitro* protein kinase activity associated with Thy-1 and Ly6.A2 (Figure 6). In these clones Ly6.A2 expression increased in parallel with the level of GPI-TIMP expressed. Clone 2.8, which was Geneticin resistant but had lost GPI-TIMP expression, had the same amount of cell surface Ly6.A2 as the parental, non-transfected cells (Figure 5b). In contrast, in clone 2.7, as in clone 2.6, Ly6.A2 increased 5-fold whereas in clone 2.9, which has less GPI-TIMP, Ly6.A2 increased only 2-fold (Figure 5b). Ninety percent of the Ly6.A2 could be released by phosphoinositide-specific phospholipase C from the surface of both untransfected and transfected cells (flow cytometry results not shown).

In each line of transfected cells, Ly6.A2 was not as much dissociated from protein tyrosine kinase activity as Thy-1 (com-

Figure 6 Autoradiography of phosphotyrosine proteins ^{32}P -labelled *in vitro* in immune complexes separated by 9% SDS/PAGE from NP40 lysates of different clones of GPI-TIMP transfected BW 5147 cells and one FACS-sorted population and one clonal population of GPI-CR1 transfected cells

Cell clones and precipitating antibodies are written above each lane. The symbol +/+ denotes the non-transfected parental BW 5147 thymoma cells.

pare Figure 2a, lanes 3 and 4 with Figures 2b and 2c, lanes 3 and 4; and likewise the corresponding antigen/antibody lanes in Figure 6 for FACS 2, clones 5, 6 and 7 with clones 8 and 9). The Thy-1 expression, however, in all of the cell sub-lines was comparable (Figure 5c). The *in vitro* phosphorylation of the protein of M_r 90000 was decreased considerably in Thy-1 immune complexes isolated from cells which expressed high levels of recombinant GPI-TIMP. Tyrosine phosphorylated proteins of M_r 59000 (which may be one of the *src* family of protein tyrosine kinases, possibly *lck*), and of M_r 40000 were also reproducibly associated with the Thy-1 and Ly6.A2 immune complexes which were highly phosphorylated *in vitro*.

GPI-TIMP was not associated with protein tyrosine kinases in any of the different clonal progeny from cells transfected with GPI-TIMP (Figure 6, lanes marked TIMP). Additionally, a different clone (35.D5) of GPI-CR1 transfectants and a FACS-sorted population of GPI-CR1 high expressors were investigated with respect to the association of GPI-CR1 with protein tyrosine kinases. The two different clones of GPI-CR1 transfected cells, 19.B6 and 35.D5 and the FACS-sorted population had very similar profiles of GPI-CR1-expressing cells by flow cytometry analysis (results not shown). Again, as had been found for clone 19.B6 (above), no association of the engineered GPI-CR1 protein with protein tyrosine kinases was found for clone 35.D5 or the FACS-sorted cell population (see the last two lanes next to the marker lane in Figure 6).

DISCUSSION

The results show a change in association and activity of protein tyrosine kinases with the natural GPI-linked proteins Thy-1 and Ly6.A2 when foreign recombinant GPI-linked proteins are

transfected into the thymoma cell line BW 5147. The introduced chimaeric GPI proteins, however, do not themselves associate with active protein tyrosine kinases.

The *src* family of protein tyrosine kinases can autophosphorylate and their activity is controlled (reviewed in Samelson and Klausner, 1992; Rudd, 1990) by the phosphorylation of the tyrosine nearest to the carboxy terminus, phosphorylation of which is inhibitory and is due to another kinase, p50 *csk* (Bergman et al., 1992). Autophosphorylation of the tyrosine in the catalytic domain is activating. *In vitro* phosphorylation of proteins associated with immune complexes reflects only the number of free tyrosine substrates available to the kinases. (The proteins may also be phosphorylated on serine/threonine residues.) Because the proteins are selected by a monoclonal antibody to phosphotyrosine, the experiments reported therefore show up differences in the phosphorylation state of the protein tyrosine kinases and their substrates in immune complexes. It can be concluded that since there was no ^{32}P -labelling of proteins in immune complexes of the recombinant chimaeric GPI-linked proteins, GPI-TIMP and GPI-CR1 do not associate with active protein tyrosine kinases as do Thy-1 and Ly6.A2 which are naturally expressed by BW 5147 thymoma cells.

Moreover, high expression of the foreign GPI proteins has decreased the association of active tyrosine kinases with Thy-1 in particular and also with a tyrosine-phosphorylated protein of M_r 90000. A protein of similar characteristics has been reported by others to be associated with Thy-1 (Stefanova et al., 1991; Thomas and Samelson, 1992) and Ly.6A (Stefanova et al., 1991) in NP40 lysates from BW 5147 thymoma cells and with Thy-1 from EL-4 cells (Stefanova et al., 1991), as has a 40000 M_r phosphotyrosine protein, also observed in this report. Octyl glucoside lysis disrupts the associations and results reported herein are in agreement. Decay-accelerating factor (a natural GPI-protein), but not an engineered transmembrane version, also associates with a phosphorylated protein of M_r 85000 to 95000 in mouse EL-4 cell NP40 lysates (Shenoy-Scaria et al., 1992).

In rat thymocytes a phosphotyrosine protein of M_r 85000 associates with Thy-1 and its *in vitro* phosphorylation is decreased with cell activation by concanavalin A (Garnett et al., 1993). The protein which is of unknown identity, could be the same as the 90000 protein discussed in this paper whose *in vitro* phosphorylation is decreased in Thy-1 immune complexes from GPI-TIMP transfected cells. The latter cells may be in a higher activation state than the parental cell line as they have more Ly6.A2 on the cell surface and, in dense cultures, they grow in large clumps. Ly6.A/E expression is up-regulated on T cell activation (Malek et al., 1989). BW 5147 cells do not express CD3, but they can still be activated to produce lymphokines when stimulated with calcium ionophore and phorbol ester (Hagiwara et al., 1988).

Possibly the high expression of competing recombinant GPI proteins could displace Thy-1 from the glycosphingolipid-rich microdomains so removing it from interactions with tyrosine kinases. Not all of the CD59 (a highly expressed natural GPI protein) on the surface of haemopoietic cells can be found in the lipid-protein complexes thought to represent these microdomains (Cinek and Horejsi, 1992), showing that it is possible for GPI proteins to be outside of the microdomains but still be on the cell surface. Preclearing the large lipid-protein complexes with antibodies to natural GPI proteins other than CD59 removed all p56 *lck* and CD59, demonstrating that all these proteins are together in the complexes (Cinek and Horejsi, 1992). High expression of GPI-TIMP should result in its efficient competition with Ly6.A2 and Thy-1 for residency in the microdomains, allowing its own interaction with tyrosine kinases but lowering each natural GPI

protein's association with the kinases to an equal degree. Because such a result was not obtained, all GPI proteins cannot be associated with non-receptor tyrosine kinases merely by virtue of their location in a common lipid-protein complex; a more specific mechanism must be invoked.

Alternatively, the six amino acids left of the human Thy-1 on the recombinant proteins (which are exactly the same six as in mouse Thy-1) and the glycan tail could specifically interact with some component of the cell surface producing a more profound change in Thy-1 occupancy of its usual site compared with that of Ly6.A2, which has a different amino acid sequence just before the GPI anchor. Such a hypothesis invokes the presence of a specific adaptor transmembrane protein, linking Thy-1 on the cell exterior with the non-receptor tyrosine protein kinase on the inner side of the plasma membrane, which could also interact with the foreign GPI proteins. This explanation, however, does not fully agree with all the data presented here because, since the engineered GPI proteins are not associated with a tyrosine kinase, they cannot be recognized by the Thy-1 specific adaptor protein.

In the *in vitro* phosphorylated immune complexes from the BW 5147 thymoma cells there is always a tyrosine-phosphorylated protein with an M_r of about 56000 to 60000. This protein may be one of the *src*-related kinases such as *fyn* or *lck* which would be responsible for the *in vitro* phosphorylations of itself and the other phosphotyrosine proteins labelled by ^{32}P in these experiments. Others have isolated the lipid-protein complexes containing GPI proteins from HPB-ALL cells (a human thymoma cell line) and found that only a small percentage of the total cellular p56 *lck* kinase is in the complexes (Cinek and Horejsi, 1992). Moreover, this last fraction has a higher specific activity than the majority of p56 *lck* and a slightly retarded mobility on SDS/PAGE. It is possible that this small fraction of *lck* is palmitoylated (which modification is both post-translational and reversible) (Paige et al., 1993) which might allow its association with the complexes. Non-palmitoylated p56 *lck* or p59 *fyn* made by site-directed mutagenesis are not immuno-co-precipitated with GPI-anchored proteins in Triton X-100 lysates from transfected cells (Shenoy-Scaria et al., 1993). The fact that only a small percentage is associated with GPI-linked proteins precludes an insufficiency of *src*-related kinases as an explanation for their lack of association with Thy-1 in lysates from cells expressing large quantities of the foreign transfected GPI-TIMP. Possibly palmitoylation of p56 *lck*, assuming that this modification might allow its transport into the sphingolipid-rich microdomains, could be rate limiting when GPI proteins are over-expressed. This hypothesis still does not explain the hierarchy in kinase association, with Ly6.A2 dominant over Thy-1 in the results presented herein.

Certainly from the work of others (Garnett et al., 1993) it appears that the association of individual, natural GPI-linked proteins with activated tyrosine kinases can alter with the cell's activation state. Whether this change is due to shifts in occupancy of glycosphingolipid-rich microdomains by the GPI-linked proteins is not known. It is possible that the putative adaptor (or bridging) molecule linking Thy-1 with *lck* or *fyn* in T cells is the phosphoprotein of M_r 90000 reported on in this paper. This protein might change its affinity for Thy-1 with its phosphorylation state which is determined, in turn, by the activity of an *src*-related kinase. A candidate protein of M_r 85000, which is phosphorylated *in vivo* and can be labelled with ^{125}I in intact cells (showing it to be a cell surface protein), is associated with Thy-1 in NP 40 lysates of mouse T cells (Bourguignon et al., 1986). My results could be explained by the existence of a family of phosphoprotein adaptors (of M_r 85000 to 95000), each

interacting with a specific GPI-linked protein, with which foreign recombinant GPI-linked proteins cannot interact fully, so excluding them from association with active tyrosine kinases. Additionally, the results reported in this paper are compatible with the supposition that the activation state of the transfected cells (as indicated by higher expression of Ly.6A2) may affect the activity and association of a non-receptor protein tyrosine kinase with Thy-1.

In summary, the reasons for the non-association of recombinant GPI-linked proteins in lysates of transfected cells with protein tyrosine kinases as found in these experiments are not known but will be investigated further. Future work will use specific antibodies to *fyn* and *lck* to measure these kinases's associations with natural and engineered GPI proteins in lysates of transfected cells and in isolated glycosphingolipid-rich microdomains. The use of cells transfected with recombinant GPI-linked proteins which are not normally on the cell surface (TIMP) or are normally transmembrane (CR1) to investigate the function of GPI anchorage is a hitherto unexplored field which should be very informative.

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