A cDNA construct of tissue inhibitor of metalloproteinases (TIMP) linked to the last exon of Thy-1 confers glycosphospholipid anchorage on this naturally secreted protein

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A naturally secreted protein, tissue inhibitor of metalloproteinases (TIMP), has been transiently expressed on the surface of transfected COS cells and stably on transfected murine BW 5147 thymoma cells, by linkage of the entire coding sequence of the cDNA to the last exon of Thy-1. Thy-1 is a glycosphospholipid-linked protein. In COS cells the chimaeric protein can be labelled by [³H]ethanolamine, which is a component of glycosphospholipid anchors. Ltk⁻ cells cannot anchor proteins by glycan phosphatidylinositol linkage and were found to be unable to express the engineered protein extracellularly on their plasma membranes. Phosphatidylinositol-specific phospholipase C treatment released 90 % of the protein from all BW 5147 cells, but very little from the COS-1 cells. It is concluded that the last exon of Thy-1 has conferred the property of glycosphospholipid anchorage on the normally secreted protein TIMP.

INTRODUCTION

Some cell-surface proteins are attached to the outer lipid bilayer of the plasma membrane through a glycosylphosphatidylinositol group. The reason for this mode of attachment is unknown, and the wide spectrum of proteins now proved to be glycosphospholipid-anchored (reviewed by Ferguson & Williams, 1988) does not provide any obvious clues as to its function. The list includes such diverse proteins as hydrolases (acetylcholinesterase, 5'-nucleotidase and alkaline phosphatase), Thy-1 (a protein of undetermined function), CD16, the receptor for immunoglobulin complexes on neutrophils, CD59 and decay-accelerating factor (DAF), which control membrane deposition of complement components and many adhesion molecules involved in cell-cell interactions. In addition the surface coats at the blood-borne stage of many parasites such as trypanosomes are attached by glycosylphosphatidylinositol linkages.

In some cases a membrane protein can be expressed in two forms, either as an intrinsic membrane protein possessing a transmembrane hydrophobic domain or as a glycosphospholipid-linked form. Examples are N-CAM (Cunningham *et al.*, 1987), LFA-3 (Dustin *et al.*, 1987; Seed, 1987) and CD16 (Selvaraj *et al.*, 1988). In the case of N-CAM it is clear that the two different forms of cell-surface linkage are regulated by alternative splicing of the N-CAM gene transcript (Cunningham *et al.*, 1987). A single exon, which is spliced out from the transmembrane-coding forms of the mRNA, encodes a protein domain which confers glycosphospholipid anchorage on N-CAM. Whether any additional information in the common exons upstream coding for the extracellular portion of N-CAM might be required is not known.

Functional protein domains can (but do not always) correspond to individual exons (Traut, 1988). In view of the N-CAM data it seemed reasonable to suppose that the last exon of Thy-1 might contain all the information required for linking a secreted protein to the cell surface by a glycosphospholipid anchor. The exon encodes the cysteine residue to which the glycosphospholipid anchor is attached (Seki *et al.*, 1985a) and a

further five amino acids to the N-terminal side, plus all 31 carboxyamino acids which are replaced by the glycosphospholipid anchor (Tse *et al.*, 1985). The coding region of the last exon of Thy-1 shows 90 % sequence identity among its mouse, rat and human counterparts, and in addition the intron/exon boundaries are conserved (Seki *et al.*, 1985a,b; Gignere *et al.*, 1985).

In order to test the 'domain hypothesis' and in the future to be able to study the function of the glycosphospholipid anchor in isolation from possible secondary interactions with other cell-surface proteins, we decided to express a hybrid protein constructed from the entire sequence of a small secreted protein, tissue inhibitor of metalloproteinases (TIMP) (Docherty *et al.*, 1985) linked to the last 37 amino acids of Thy-1 (encoded by the gene's last exon). Thy-1 is advantageous to test the hypothesis that the protein domain conferring glycosphospholipid anchorage is encoded in a single exon, by analogy with exon switching in the N-CAM and acetylcholinesterase genes (Gibney & Taylor, 1990), because both the amino acid to which the anchor is attached and the intron-exon structure of the gene are known. The site of attachment of the anchor for other proteins studied such as DAF, Qa-2 and CD16 has only been surmised, although these proteins have been used to study requirements for anchorage. Very recently, however, the site in DAF has been published (Moran *et al.*, 1991). The aim of the present study was to engineer a construct capable of directing cell-surface expression of a normally secreted protein linked artificially to a glycosphospholipid anchor, but which retains the minimum possible sequence of the parent protein conferring glycosphospholipid anchorage. The cDNA construct will allow us to study the functional consequences of attaching a proteinase inhibitor to the cell surface by a glycosphospholipid anchor.

MATERIALS AND METHODS

Plasmids

The expression vector used in this study was modified from the pSV2 series (Subramani & Southern, 1983) and is shown in Fig. 1. The simian-virus-40 (SV40) ori/early promoter enables the

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; PI-PLC, phosphatidylinositol-specific phospholipase C; DAF, decay-accelerating factor; SV40, simian virus 40; FACS, fluorescence-activated cell sorting; HBSS, Hanks buffered saline solution; PBS, phosphate-buffered saline (7.5 mM-sodium/potassium phosphate/0.137 M-NaCl, pH 7.2); FITC, fluorescein isothiocyanate.

Fig. 1. Eukaryotic expression plasmid with the hybrid TIMP–Thy-1 sequence

In the box below the plasmid map is the amino acid sequence of the last exon of Thy-1. The cysteine residue to which the glyco-phospholipid anchor is attached *in vivo* is underlined. Abbreviations used in the plasmid map: *Amp^r*, ampicillin-resistance gene; SV40 Ori, simian-virus-40 origin of replication; *ColI* Ori, colicin origin of replication; CMV IE promoter, cytomegalovirus immediate early promoter; *HYGRO*, hygromycin-resistance gene; *HindIII* (3897) etc. are cleavage points of various restriction endonuclease (numbers refer to bases).

plasmid to replicate in COS cells for transient expression and to express hygromycin resistance for establishment of stable cell lines. The immediate early cytomegalovirus promoter was cut out from the vector *pH3* (Aruffo & Seed, 1987) and used to drive the expression of the hybrid protein TIMP–Thy-1. The polyadenylation signal operates in both directions and includes the early and late SV40 polyadenylation signals. A chloramphenicol acetyl transferase version of this plasmid was also constructed to check its efficiency in COS cells and other cell lines (results not shown). The cDNA clone for TIMP was obtained from a human fibroblast cDNA library (P. M. Clissold, unpublished work) cloned by the Okayama & Berg (1982) method in their non-expression version. The last exon of Thy-1 was excised from an 8.3 kbp human genomic clone of Thy-1 (Seki *et al.*, 1985b) on a *PstI*–*ApaI* 125 bp fragment. Its identity was confirmed by sequencing in the Stratagene vector KS+. The genomic clone was kindly given by Dr. J. Silver. The T7/SP6 vector was pAM19 from Amersham International. The CD2 plasmid used as a positive control for transfections is Seed & Aruffo's (1987) construct in *pH3*.

For stable transfection, murine BW 5147 thymoma cells were co-transfected with the pSV2 NEO plasmid and a plasmid derived from *pEI* CAT (Dr. G. Wilkinson). This vector contains a splice site after the test gene but before the polyadenylation site. The CAT gene was replaced with the same insert as shown in Fig. 1 and described in the Results section.

Cells

COS-1 cells, murine BW 5147 thymoma cells and Ltk⁻ cells were routinely maintained in DMEM (Dulbecco's modified Eagle medium) plus 10% (v/v) foetal-calf serum and antibiotics.

Photography of cells was done using the Bio-Rad 500 MRC microscope.

Transfections

COS and Ltk⁻ cells were transfected by the DEAE-dextran method of Seed & Aruffo (1987). After 24 h the cells were trypsin-treated and removed to new flasks. In some experiments a percentage of cells were re-plated on Lab-Tek eight-well glass slides. At 48 h after transfection, cells were removed with 1 mM-EDTA in Hanks balanced salt solution (HBSS) for fluorescence-activated-cell-sorting (FACS) analysis. Cells growing on slides were immunostained *in situ* and fixed with 4% (w/v) para-formaldehyde (w/v) in phosphate-buffered saline (PBS) at 4 °C after staining. Antifade (Citifluor) was used as mounting medium.

BW 5147 cells were transfected by electroporation using a Bio-Rad Gene Pulser delivering 250 V and 960 μ F (0.4 cm electrode). Cells (5×10^6) were suspended in 660 μ l of Hepes/saline (Potter, 1988) containing 40 μ g of pSV2 NEO and 20 μ g of the TIMP–Thy-1 construct in the vector *pEI*. After 48 h Geneticin (Gibco) at 2 mg/ml was added. The selection drug was increased to 6 mg/ml on day 9, and on day 12 dead cells were removed with a Nycodenz step gradient (Nycomed AS, Oslo, Norway). Cells were FACS-sorted (Becton–Dickinson FACSTAR) on day 14, and positive cells expressing surface TIMP (4% of the total) were grown on in Geneticin at 6 mg/ml. Clones of positive cells were obtained by the method of limiting dilution.

Phase separations

Phase separation with Triton X-114 was as described by Bordier (1981). Cells were lysed with 60 mM-octyl glucoside (Hooper & Turner, 1988) in 150 mM-NaCl/25 mM-Tris/HCl, pH 8.0, at 0 °C. The proteinase inhibitors leupeptin and pepstatin both at 10 μ g/ml were included in the lysis buffer with 2 mM-EDTA. DNA and other insoluble material was pelleted at 10000 *g* at 0 °C. Triton X-114 was added to 1%, and the octyl glucoside was removed by dialysis at 0 °C. The dialysis residue was phase-separated at 37 °C and proteins in the detergent phases and aqueous phases were trichloroacetic acid-precipitated. The pellets were washed in ice-cold acetone and were then boiled in Laemmli reducing buffer before applying to an SDS/15% polyacrylamide gel (Laemmli, 1970). Protein molecular-mass markers were ¹⁴C-labelled Rainbow markers (Amersham International). Gels were fluorographed using Amersham's Amplify.

Antibodies

The mouse monoclonal antibodies to TIMP, namely MAC 015 and MAC 019 (Cockett *et al.*, 1990), purified from cell culture media, were kindly provided by Celltech. The monoclonal antibody to CD2 was T11 bought from Dakopatts and used as recommended. The second, fluorescein isothiocyanate (FITC)-conjugated antibody was anti-mouse IgG from Sigma.

Flow cytometry

Cells were plated at a density of 1×10^4 cm² 16 h before transfection. After 24 h, cells were trypsin-treated and transferred to fresh flasks (25 cm²). At 48 h after transfection cells were removed with 1 mM-EDTA in HBSS, each transfected sample divided into two and incubated with or without 2 μ l of phosphatidylinositol-specific phospholipase C (PI-PLC). The PI-PLC was kindly given by Dr. Martin Low and was used at 1:250 dilution at 37 °C for 20 min (the original batch activity was 325 μ mol/min per ml assayed by hydrolysis of [³H]phosphatidylinositol). A 0.5 μ l portion was sufficient to release 90% of Thy-1 from 10^6 murine BW 5147 thymoma cells (results not shown). Cells were incubated on ice for 1 h with monoclonal antibody,

Fig. 2. Flow-cytometric analysis of surface TIMP on COS cells 48 h after transfection

Cells were plated at a density of 1×10^4 cm² 16 h before transfection. Cells were transfected with 5 μ g of pSV2 NEO DNA (a and d) or 2 μ g of TIMP–Thy-1 expression vector plus 4 μ g of pSV2 NEO DNA, at a molar ratio of 1:3 (b, c, e and f). After 24 h, cells were trypsin-treated and replated in fresh flasks (25 cm²). At 48 h after transfection, cells were removed with 1 mM-EDTA in HBSS; each transfected sample was then divided into two and incubated with or without 2 μ l of PI-PLC. The first antibody, MAC 015 (b and c) or MAC 019 (e and f) was at 4.5 μ g/ml, and the second FITC-conjugated anti-(mouse IgG) antibody at 2 μ g/ml. Incubations were done on ice for 1 h each. Propidium iodide at 0.5 μ g/ml was included in the final wash. In each series of analyses the background was taken as the percentage of positive cells transfected with pSV2 NEO alone (a and d). '% Gated' means the percentage of cells showing positive fluorescence. FL1 is fluorescence due to FITC labelling in arbitrary units on a logarithmic scale.

then washed at 0 °C and incubated with FITC-conjugated second antibody for a further 1 h at 0 °C. For FACS analysis cells were treated with propidium iodide at 0.5 μ g/ml in the last wash. Cells were analysed with a Becton–Dickinson FACSCAN using four parameters, namely forward scatter, side scatter, fluorescence from FITC and fluorescence from propidium iodide. In each series of analyses the background was taken as the percentage of positive cells transfected with pSV2 NEO alone.

Immunoprecipitation

For precipitation by antibody, cells (1×10^6) were lysed in 1 % Triton X-100/150 mM-NaCl/25 mMTris/HCl (pH 8.0)/2 mM-EDTA with proteinase inhibitors leupeptin and pepstatin at 10 μ g/ml each and 200 μ M-phenylmethanesulphonyl fluoride. Cells were first detached by 1 mM-EDTA in HEPES-buffered saline and were then left in lysis buffer for 1 h at 0 °C. Insoluble material was pelleted at 10000 g, and 10 μ g of the monoclonal MAC 015 was added to the supernatant, which was then incubated for a further 3 h at 0 °C. The pH was lowered to 5.2 with 6 M-acetic acid and protein G beads were added and incubation was continued for a further 2 h on a rotating wheel at 0 °C. Proteins bound to the beads were separated from unbound protein by collection of beads on Costar Spin-x filters. Beads were washed in 10 % Triton X-100 before addition of Laemmli loading buffer containing 2 % SDS and mercaptoethanol. After boiling the supernatant was electrophoresed on a 15 % polyacrylamide Laemmli gel. The unbound proteins in the filtrate were precipitated with trichloroacetic acid. The precipitate was washed with ice-cold acetone and was dissolved in Laemmli loading buffer ready for electrophoresis.

Translation in vitro

Capped mRNA was transcribed from TIMP–Thy-1 in pAM19 using SP6 polymerase and 0.5 mM of each ribonucleoside plus 0.5 mM-7mG(5')ppp(5')G according to the Promega Protocols and Applications Guide. If required, canine pancreatic membranes (Promega) were added to the reticulocyte lysate before addition of the mRNA. Translation of mRNA was as recommended by Promega.

Enzymes and special reagents

Purified PI-PLC was a gift from Dr. Martin Low and was isolated from *Bacillus thuringiensis*. SP6 polymerase and 7m(G')ppp(5')G was from New England Biolabs. RNasin, reticulocyte lysate and canine pancreatic microsomal membranes were from Promega. Ribonucleosides were from Pharmacia. Leupeptin and pepstatin were from Boehringer. Protein G beads were from Pierce.

RESULTS

Construction of expression clone

The entire cDNA coding sequence of TIMP (Docherty *et al.*, 1985) was linked in phase to the last exon of the human Thy-1 gene. The only change made to the TIMP sequence was the replacement of the C-terminal alanine residue with a proline residue to provide a bulky helix-breaking amino acid. This proline codon was joined in phase (confirmed by sequencing) to the first amino acid codon (aspartate) of the last exon of Thy-1. The resulting hybrid cDNA sequence codes for the entire TIMP

Fig. 3. [^3H]Ethanolamine labelling of transfected COS cells at 48–64 h after transfection

Cells (1×10^6) were labelled overnight with 250 μCi of [^3H]ethanolamine (Amersham International; 19 Ci/mmol) in 5 ml of medium. Cells were lysed with octyl glucoside and were then Triton X-114-phase-separated as described in the Materials and methods section. The exposure time of the fluorographed gel was 2 weeks at -70°C . Lanes A–D, extracts from COS cells transfected with: A, pSV2 NEO plasmid, detergent phase; B, TIMP–Thy-1 plasmid, detergent phase; C, pSV2 NEO plasmid, aqueous phase; D, TIMP–Thy-1 plasmid, aqueous phase. Lane M contains molecular-mass markers.

Fig. 4. Antibody precipitation of protein from [^3H]ethanolamine-labelled transfected COS cells

Transfected COS cells (1.5×10^6) were labelled from 26 to 46 h after transfection with [^3H]ethanolamine (40 $\mu\text{Ci}/\text{ml}$; 29.5 Ci/mmol; Amersham International). Lanes A and B show [^3H]ethanolamine-labelled proteins which bound to antibody and protein G beads: lane A, TIMP–Thy-1-transfected COS cells; lane B, pSV2 NEO-transfected COS cells. Lane M contains marker proteins.

protein joined to the last 37 amino acids of Thy-1. The construct is shown in Fig. 1. During processing of native Thy-1 *in vivo* the last 31 amino acids are removed and replaced by a glycopospholipid anchor attached through an amide bond to the terminal cysteine residue (Homans *et al.*, 1988). If this pathway is followed by the hybrid TIMP–Thy-1 protein, only six amino acids of Thy-1 will remain, 31 amino acids having been replaced by a glycopospholipid anchor. The hybrid protein should be expressed on the cell surface attached by a glycopospholipid anchor and should not be secreted into the medium as is native TIMP.

Fig. 5. Translation of TIMP–Thy-1 mRNA *in vitro*

Capped mRNA was transcribed from TIMP–Thy-1 in pAM19 as described in the Materials and methods section. [^{35}S]Methionine (1 mCi/ml) was used to label ‘*in-vitro*’-translated TIMP–Thy-1 protein in 20 μl of reticulocyte lysate in the presence (+) or absence (–) of canine pancreatic microsomal membranes (2 μl ; Promega). The control (c) was without any SP6 transcript. Aliquots (5 μl) were diluted with twice concentrated Laemmli reducing buffer, boiled, and run on a 15%-polyacrylamide Laemmli gel. The gel was fluorographed, the exposure time being 4 days at -70°C . The sharp band at about 50 kDa is due to an activity in the membranes.

Preliminary transfection experiments

Transfected COS cells were assayed for surface TIMP expression by surface immunostaining and FACS analysis 48 h after transfection with the TIMP–Thy-1 hybrid plasmid. In all experiments a portion of COS cells was transfected with a human CD2 expression plasmid (Seed & Aruffo, 1987) as a positive control to monitor the efficiency of the transfection procedure (results not shown for COS cells). Usually about 15–20% of the COS cells expressed CD2 (an intrinsic membrane protein). Two anti-(human TIMP) mouse monoclonal antibodies, namely MAC 015 and MAC 019 (kindly given by Celltech), were used to detect expression of TIMP on the cell surface by FACS analysis (Fig. 2). In preliminary experiments, expression from the plasmid was found to be so high that it appeared to be somewhat toxic to the cells. When the plasmid was diluted 4-fold with pSV2 NEO and transfected into COS cells, the cell viability was improved and expression of surface TIMP was not greatly lowered (results not shown). Both mouse monoclonals recognized a high level of surface TIMP (Figs. 2b and 2e) that was not present on control pSV2 NEO-transfected COS cells (Figs. 2a and 2d).

Glycopospholipid-linked proteins can be removed from some cell membranes by a bacterial enzyme, PI-PLC, that cleaves between the phosphate and the lipid tails leaving diacylglycerol in the membrane and releasing the protein still attached to the glycan inositol group through an ethanolamine phosphate moiety (reviewed by Low, 1989). On other cells, however, such as the human erythrocyte, the inositol ring is modified by a palmitoyl chain which blocks the action of PI-PLC, making the protein resistant to release by the enzyme (Toutant *et al.*, 1989). When the COS cells were treated with PI-PLC just before immunostaining with MAC 015, the percentage of TIMP-positive cells decreased from 13.9 to 11% (Figs. 2b and 2c). The percentage decrease in positive cells in three separate experiments was between 20 and 23% as detected by the monoclonal MAC 015, but no decrease could be detected with MAC 019. (MAC 019

Fig. 6. Indirect fluorescent-antibody staining of COS cells in situ 60 hours after transfection

The left-hand side of each Figure is the phase-contrast picture and the right-hand side is its fluorescent counterpart. As the expression is transient, only about 15% of the cells are immunolabelled, as found in Fig. 2 by FACS analysis. The Bio-Rad 500 MRC confocal microscope sees the cells as an 'optical section'. (a) Cells fixed in 3.5% paraformaldehyde/PBS at 0 °C for 30 min after immunofluorescent staining. (The bar represents 25 μm .) (b) Cells fixed before staining (same magnification as a).

recognizes a different epitope on TIMP from the one bound by MAC 015; C. Bailey, personal communication). Whether this apparent difference in recognition between MAC 015 and MAC 019 could be real, signifying different conformational states of surface TIMP on COS cells that correlate with its sensitivity to PI-PLC cleavage, remains to be investigated.

Characterization of the chimaeric protein made in COS cells

Glycophospholipid-linked proteins may be specifically labelled by incorporation of radioactive ethanolamine into the ethanolamine of the anchor group. COS cells at 48 h after transfection were labelled with [^3H]ethanolamine for 16 h. Cell lysates were phase-separated with Triton X-114 (Bordier, 1981) as described in the Materials and methods section, and the molecular masses of [^3H]ethanolamine-labelled proteins were determined. A faint diffuse band at about 32 kDa could be seen in the TIMP–Thy-1-hybrid-transfected cells, but not in the control cells transfected only with pSV2 NEO (Fig. 3). The band (arrow in Fig. 3) was in the detergent phase, as expected for a protein linked to a lipid moiety. The 32 kDa band labelled by [^3H]ethanolamine is precipitated from Triton X-100 lysates by the TIMP MAC 015 monoclonal antibody bound to protein G beads (Fig. 4). The very heavily labelled band at 46 kDa has been shown to be the protein-synthesis factor Elongation Factor 1 α (Rosenberry *et al.*,

Fig. 7. Flow-cytometric analysis of transfected Ltk⁻ cells 48 h after transfection

(a and c) Ltk⁻ cells transfected with 5 μg of pSV2 NEO (controls), incubated with monoclonal antiserum to CD2 (a) and MAC 015 (c); (b) Ltk⁻ cells transfected with 5 μg of pCD2 and incubated with monoclonal antiserum to CD2; (d) Ltk⁻ cells transfected with 5 μg of TIMP–Thy-1 and incubated with the monoclonal MAC 015. Transfection methods were as described in the Materials and methods section, except Ltk⁻ cells were plated at three times the density of COS cells. Conditions for incubation of cells with antiserum were as for Fig. 2. The second FITC-conjugated antibody [anti-(mouse IgG) antibody; Sigma] was the same for all experiments, including CD2. CD2 antiserum was the T-11 mouse monoclonal (Dakopatts) and used as recommended. '% Gated' and FL1 are explained in the legend to Fig. 2.

1989). The band partitions into the aqueous phase of Triton X-114 separations (Fig. 3), as would be expected for a cytosolic protein.

Size of the chimaeric protein synthesized *in vitro*

To determine the approximate size of the glycosylated mature TIMP hybrid protein, the hybrid cDNA sequence was inserted into an SP6 vector and transcribed into capped messenger RNA by SP6 polymerase *in vitro*. The mRNA was then translated into ^{35}S -labelled protein in a reticulocyte system in the presence or absence of endoplasmic-reticulum membranes (Fig. 5). A protein of 27 kDa was made without membranes, as expected from the molecular mass of TIMP (22 kDa) plus 37 amino acids of Thy-1 (4.7 kDa). When membranes were present during translation, glycosylation increased the molecular mass of the hybrid protein to 32 kDa, and the band became diffuse, indicating variation in the amount of non-core glycosylation. The molecular mass of natural secreted TIMP synthesized in a similar '*in vitro*' translation system was 28 kDa, which is the same as its molecular mass *in vivo* (Waterhouse *et al.*, 1990).

The molecular mass of 32 kDa correlated well with that of the band labelled by ^3H -ethanolamine in the TIMP–Thy-1-transfected COS cells. Replacement of the last 31 amino acids of Thy-1 by a glycophospholipid anchor will make only a small difference in molecular mass and an unpredictable change in mobility on an SDS/polyacrylamide gel. Immunoprecipitation with monoclonal antibody to TIMP confirmed that the 32 kDa band labelled by ethanolamine was the TIMP–Thy-1 chimaeric protein.

Cellular localization of the chimaeric protein

Cells 24 h after transfection were passaged on to glass microscope slides for indirect fluorescent-antibody staining *in situ* at

Fig. 8. Flow-cytometric analysis of surface TIMP on a clone of BW 5147 cells stably transfected with the plasmid pEI expressing the TIMP-Thy-1 chimaeric protein

(a) Cells incubated with second antibody alone; (b) cells incubated with MAC 015 monoclonal antibody ($3 \mu\text{g}$ in 0.5 ml) and then second antibody; (c) cells treated with PI-PLC (see the Materials and methods section) before incubation with antibodies in (b). FL1 is explained in the legend to Fig. 2.

60 h after transfection. In unfixed cells (stained at 0°C) the surface TIMP appeared in small patches under the confocal microscope (Fig. 6a), whereas in fixed cells the surface TIMP was diffuse, but still particulate, especially in some areas (Fig. 6b). The percentage of positive cells staining agreed with the flow-cytometry results and there was no non-specific staining. Transfected cells treated with PI-PLC while attached to the microscope slide, rather than being treated in suspension after detachment by EDTA, still did not release the TIMP-Thy-1 protein (results not shown).

Is TIMP expressed on a glycopospholipid anchor? Expression by Ltk⁻ cells

To test further whether TIMP was expressed on a glycopospholipid anchor, the TIMP-Thy-1 expression plasmid was transfected into Ltk⁻ cells. These cells cannot anchor proteins by a glycopospholipid group. The positive control was again CD2 in $\pi\text{H}3$ which uses the same promoter as the TIMP-Thy-1 plasmid to drive CD2 expression. At 48 h after transfection, 7%

of the Ltk⁻ cells expressed CD2 on their surface (Figs. 7a and 7b), whereas there was no surface expression of TIMP (Figs. 7c and 7d). MAC 015 recognizes TIMP secreted by both primate cells and Chinese-hamster ovary cells (Cockett *et al.*, 1990), and so the use of non-primate cells (i.e. rodent) should not affect the antibody's recognition of TIMP.

A stable expression system in cells which are known to release glycopospholipid anchored proteins with PI-PLC

The Thy-1 on murine BW 5147 thymoma cells is very sensitive to release by PI-PLC, and so the chimaeric TIMP-Thy-1 plasmid was electroporated into the thymoma cells for stable expression of cell-surface TIMP and to test its sensitivity to PI-PLC. After 2 weeks in selection medium the cells were FACS-sorted to remove cells which were Geneticin-resistant but surface-TIMP-negative. After a further 2 weeks culture in Geneticin-containing medium, the cell population was retested for surface TIMP expression; 90% of the cells were positive with both monoclonals (MAC 015 and MAC 019). PI-PLC treatment before incubation with antibodies decreased the mean surface fluorescence by 90%. The cells were then cloned and after a further 4 weeks in culture (8 weeks after electroporation) in Geneticin, individual clones were re-tested for TIMP membrane expression and its release by PI-PLC. FACS analysis of one such clone showed that 85% of the cells were highly positive for surface TIMP, which was decreased 9-fold on all of the positive cells by PI-PLC treatment. These cells were then recloned and sub-clones were obtained which were 100% positive for surface TIMP expression by FACS analysis (Fig. 8b); 90% of the TIMP was removed by PI-PLC from these cells (Fig. 8c).

DISCUSSION

Others have conducted extensive studies on the consensus sequence which determines cleavage and replacement of a C-terminal domain by a glycopospholipid anchor. All studies to date show that a hydrophobic C-terminal sequence is necessary for glycopospholipid anchorage of a protein (Caras *et al.*, 1989; Brown *et al.*, 1989) and that a minimum length of 17 amino acids is required (Berger *et al.*, 1988). The question, however, of how much of the original protein sequence upstream of the cleavage site is necessary for successful glycosylphosphatidylinositol anchorage, has not been investigated so thoroughly. The mode of anchorage of such proteins as DAF (Moran *et al.*, 1991), Qa-2 (Ulker *et al.*, 1990), CD16 (Lanier *et al.*, 1989) and alkaline phosphatase (Micanovic *et al.*, 1990), all of which are naturally glycopospholipid-linked, has been investigated after change of various amino acids by site-directed mutagenesis. Collectively, their results show that the dominant factor in glycopospholipid anchorage is the presence of a correct cleavage site (a hydrophilic amino acid or the small neutral amino acids alanine or glycine) followed, after some ten or so neutral/hydrophilic amino acids, by a stretch of hydrophobic residues which can be recognized by the putative transamidase.

Investigations (Kurosaki & Ravetch, 1989; Lanier *et al.*, 1989) on the determination of the mode of attachment of CD16 (Fc γ RIII) to the cell membrane show that exchange of serine with phenylalanine at position 203 causes the expression of CD16 to switch from the glycopospholipid-anchored form to an integral transmembrane protein and vice versa. In these studies, high surface expression of CD16 in COS cells and non-expression in Ltk⁻ cells was indicative of glycolipidation of CD16 mutants obtained by genetic engineering. Recently it has been demonstrated conclusively that Ltk⁻ cells cannot perform reactions required for the synthesis of glycolipid anchors (Singh *et al.*, 1991). The TIMP-Thy-1 hybrid protein was highly

expressed on the surface of COS cells, but not by Ltk⁻ cells. The reason for its relative resistance to removal by PI-PLC from COS cells remains unexplained for the moment. It is possible that a percentage remains as an intrinsic transmembrane protein in COS cells, but in view of the null expression in Ltk⁻ cells, this explanation is unlikely. Natural Thy-1 never appears on the cell surface as an integral transmembrane form, even in mutant cell lines which cannot perform the reactions required for anchorage by glycolipidation (reviewed by Hyman, 1988). Thy-1 isolated from the mutant classes A and E of murine BW 5147 thymoma cells does not contain the C-terminal glycopospholipid membrane anchor, but retains its original C-terminal peptide sequence (Conzelmann *et al.*, 1985). These cells, however, do not express surface Thy-1. Moreover, forms of placental alkaline phosphatase obtained by site-directed mutagenesis of aspartate-484 that cannot be recognized by the putative transamidase involved in glycolipidation are never expressed on the cell surface, but are retained intracellularly, (Micanovic *et al.*, 1990). There may be some sort of endoplasmic-reticulum-retention signal in C-terminal signal peptides which does not allow their exit to the cell surface. At first inspection it may be thought that the results from CD16 surface expression of mutated forms do not agree with the latter hypothesis until it is realized that the non-glycopospholipid-anchored version of CD16 can only be brought to the cell surface in association with a 'transport' molecule, the γ -chain of the high-affinity IgE receptor (Hibbs *et al.*, 1989; Kurosaki & Ravetch, 1989; Lanier *et al.*, 1989).

While this work was in progress, Moran *et al.* (1991) reported the amino acid site of attachment of the glycopospholipid anchor in DAF. Analysis of the genomic structure of DAF (Post *et al.*, 1990) shows that the hydrophobic C-terminal domain is encoded by a single exon, while the actual attachment site is encoded in the next-but-one preceding exon. Alternative splicing creates a different reading frame, so eliminating the last 17 contiguous hydrophobic amino acids, thus encoding a possible secreted form of DAF. Differential usage of exons produces different isoforms of the protein DAF, but the finding reported here that a single exon (of Thy-1) confers glycopospholipid anchorage is not therefore universally applicable to all glycopospholipid-linked proteins. In the example of DAF, the protein domain constituting the consensus sequence for anchorage is split into several exons. In the case of *Torpedo* acetylcholinesterase (Gibney & Taylor, 1990), N-CAM (Cunningham *et al.*, 1987) and Qa-2 (Ulker *et al.*, 1990), however, all information required for glycopospholipid anchorage resides in a single exon. Brown *et al.* (1989) have reported that when the last 53 amino acids of Thy-1 were joined to the entire extracellular portion of the vesicular-stomatitis-virus G protein, the hybrid protein was glycopospholipid linked in MDCK cells. If a shorter sequence (of unstated number of residues) was used, the protein failed to exit from the endoplasmic reticulum. Results reported herein show that 37 amino acids of Thy-1 (as opposed to 53) are sufficient to confer glycopospholipid anchorage.

The reason for non-cleavage of TIMP–Thy-1 from COS cells by PI-PLC requires further investigation. [³H]Ethanolamine labelling shows that it is attached by a glycopospholipid anchor in COS cells, as it is in the BW 5147 stable transfectants, from which it can be released by PI-PLC. The inability of PI-PLC to release a protein from the cell surface is not a sufficient criterion of its non-anchorage by a glycopospholipid group. Secondary modifications of the inositol ring by acylation can result in resistance to PI-PLC cleavage (Toutant *et al.*, 1989), but this modification is generally thought to be a property of the cell and not of the protein (Toutant *et al.*, 1990). There exist two forms of the K562 cell line, one of which modifies the anchor and one which does not. Our COS-1 cell line may modify the anchor of

glycopospholipid-linked proteins, although the modification is not usual in COS-7 cells.

It is concluded from the high surface expression of TIMP on BW 5147 and COS cells and its efficient release by PI-PLC from BW 5147 cells that the C-terminal 37 amino acids of Thy-1 (encoded solely by the last exon) are able to confer glycopospholipid linkage on a small secreted protein, TIMP. In substantiation of glycopospholipid linkage, the protein can be labelled in COS cells with [³H]ethanolamine, a component of the anchor. Additionally, the hybrid protein is not expressed in transiently transfected Ltk⁻ cells. As *in vivo*, exon shuffling in the laboratory can be used to create alternative forms of proteins. The diversity of proteins which are now known to be glycopospholipid-linked argues against any specialized extracellular domain being obligatory for this linkage to occur. It now seems possible to confer new properties by genetic engineering on secreted or transmembrane proteins by linking them to the cell surface by a glycopospholipid anchor. This mode of attachment seems to allow very-high-density surface expression without upsetting the lipid bilayer (Edidin & Stroynowski, 1990) and so may provide a 'solid-phase carriage system' for secreted proteins. The linkage may not, however, itself be inert (Pont *et al.*, 1985), which opens up many interesting avenues of research using artificially linked glycopospholipid-anchored proteins.

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