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# Comparison of the glycosyl-phosphatidylinositol cleavage/attachment site between mammalian cells and parasitic protozoa

**Ian J. White, Abdallah Souabni* and Nigel M. Hooper‡**

School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK
*Present address: Institute for Molecular Pathology, Dr Bohr Gasse 7, A-1030 Vienna, Austria
‡Author for correspondence (e-mail: n.m.hooper@leeds.ac.uk)

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## SUMMARY

**It was previously hypothesised that the requirements for glycosyl-phosphatidylinositol (GPI) anchoring in mammalian cells and parasitic protozoa are similar but not identical. We have investigated this by converting the GPI cleavage/attachment site in porcine membrane dipeptidase to that found in the trypanosomal variant surface glycoprotein 117 and expressing the resulting mutants in COS-1 cells. Changing the entire ω, ω+1 and ω+2 triplet in membrane dipeptidase from Ser-Ala-Ala to Asp-Ser-Ser resulted in efficient GPI anchoring of the mutant proteins, as assessed by cell-surface activity assays and susceptibility to release by phosphatidylinositol-specific phospholipase C. Immunoelectrophoretic blot analysis with antibodies recognising epitopes either side of the native ω residue in porcine membrane dipeptidase, and expression of a mutant in which potential alternative cleavage/attachment sites were disrupted, indicated that alternative GPI cleavage/attachment sites had not been used. These results indicate that the requirements for GPI anchoring between mammalian and protozoal cells are not as different as previously suggested, and that rules for predicting the probability of a sequence acting as a GPI cleavage/attachment site need to be applied with caution.**

Key words: GPI, Variant surface glycoprotein, Membrane dipeptidase, Transamidase

## INTRODUCTION

A diverse range of eukaryotic cell-surface proteins are anchored in the membrane through a covalently attached glycosyl-phosphatidylinositol (GPI) moiety, including protozoal surface coat proteins, receptors, adhesion molecules, differentiation antigens and enzymes (McConville and Ferguson, 1993; Ferguson, 1999). In many ways a GPI anchor can be considered primarily as an alternative to a hydrophobic transmembrane polypeptide anchor, although several additional functions have been proposed for GPI anchors. These include roles in intracellular sorting (Rodriguez-Boulan and Powell, 1992), in transmembrane signalling (Brown, 1993), and in the endocytic process of potocytosis (Anderson, 1998). A GPI anchor also enables a protein to associate with cholesterol and glycosphingolipid-rich membrane domains (Brown and London, 1998; Hooper, 1999), to be more laterally mobile in the plane of the bilayer (Ishihara et al., 1987), and to be selectively released from the membrane by phospholipases (Metz et al., 1994; Movahedi and Hooper, 1997; Solter and Hoffman, 1995).

A protein destined to be GPI anchored is translated with a cleavable N-terminal signal peptide that directs the growing polypeptide to the endoplasmic reticulum (ER), for cotranslational insertion into the ER membrane. At the C terminus of such proteins is another signal sequence, which directs addition of the preformed GPI-anchor within the lumen of the ER (Englund, 1993; Udenfriend and Kodukula, 1995a). This C-terminal signal sequence consists of a predominantly hydrophobic region of 8-20 amino acids preceded by a hydrophilic spacer region of 8-12 amino acids, which is often rich in charged amino acids and proline (Udenfriend and Kodukula, 1995b). At the N terminus of the spacer region is the site of cleavage of the polypeptide chain and attachment of the GPI anchor. Cleavage of the polypeptide chain occurs on the C-terminal side of an internal amino acid residue (termed ω) lying in a particular consensus sequence ω, ω+1, ω+2, with the concomitant addition of a preformed GPI anchor to the newly exposed COOH group of the ω residue. Analysis of native GPI-anchored protein sequences and extensive site-directed mutagenesis of these residues in alkaline phosphatase (Gerber et al., 1992; Kodukula et al., 1993), decay accelerating factor (Moran et al., 1991) and yeast Gas1 protein (Nuoffer et al., 1993) has shown that ω is restricted to amino acids with small side chains (Ala, Asn, Asp, Cys, Gly or Ser), whereas ω+1 can be any residue except for Pro, and ω+2 is usually Gly or Ala in mammalian cells or Ser in pathogenic protozoa. Rules/algorithms for predicting the site of GPI anchor addition in proteins have been devised based on these experimental observations (Udenfriend and Kodukula, 1995b; Eisenhaber et al., 1999). A transamidase enzyme, probably Gpi8p (Benghezal et al., 1996; Yu et al., 1997), is believed both to

cleave the polypeptide chain and attach the preformed GPI anchor. Other gene products, including Gaa1p (Hamburger et al., 1995; Hiroi et al., 1998), are also involved in this process.

Expression of the parasitic proteins *Trypanosoma brucei* variant surface glycoprotein (VSG) and *Plasmodium berghei* circumsporozoite protein in COS cells led to inefficient GPI processing of both proteins, leading to the hypothesis that the requirements for GPI processing in parasitic protozoa are similar but not identical to those of mammalian cells (Moran and Caras, 1994). Neither the properties of the C-terminal hydrophobic domain nor the length of the hydrophilic spacer region were responsible for the impaired activity of the VSG GPI signal in COS cells. Replacement of residues in the ω, ω+1 and ω+2 positions (Asp-Ser-Ser) of the VSG GPI signal with the corresponding residues (Ser-Gly-Thr) from mammalian decay accelerating factor converted the inactive VSG GPI signal to a viable signal for mammalian cells, leading to the conclusion that the binding pocket in the mammalian transamidase is smaller than in its trypanosome counterpart.

Membrane dipeptidase (MDP, leukotriene $D_4$ hydrolase; EC 3.4.13.19) was the first mammalian peptidase to be identified as GPI anchored (Hooper et al., 1987). Porcine MDP is an N-glycosylated, disulphide-linked homodimer of subunit molecular mass 47 kDa (Keynan et al., 1996; Littlewood et al., 1989). The cDNA derived amino acid sequence predicts a protein of 409 residues, with a cleaved N-terminal signal sequence and a characteristic C-terminal GPI anchor attachment signal (Rached et al., 1990); Ser368, Ala369 and Ala370 comprise the ω, ω+1 and ω+2 residues, there is a 9-amino-acid spacer region that contains 3 proline residues, and a hydrophobic sequence of 14 amino acids (Fig. 1). Ser368 was identified as the C-terminal amino acid in the mature protein by amino acid sequencing of the C-terminal tryptic peptide (Brewis et al., 1995). Using MDP as a model native GPI-anchored protein, we investigated whether the requirements for GPI processing in mammalian cells are indeed different to those in parasitic protozoa by altering the residues at the ω, ω+1 and ω+2 site to those in trypanosome VSG, which would be predicted to result in inefficient GPI anchoring when expressed in COS cells. Unexpectedly the mutations at and around the GPI cleavage/attachment site, including one designed to remove any alternative cleavage/attachment sites, still acted as viable GPI anchoring signals in COS-1 cells, implying that the requirements for GPI processing between mammalian and protozoal cells are not as significantly different as previously reported.

## MATERIALS AND METHODS

### Site-directed mutagenesis

Site-directed mutagenesis was performed according to the method of Kunkel et al. (1987). *Escherichia coli* CJ236 was transformed with the plasmid pEF-MDP (Keynan et al., 1994). From an ampicillin-resistant colony, single-strand DNA (containing uracil) was isolated using helper phage R408 (according to the standard protocol of Boerhinger-Mannheim). All the mutagenic primers were designed to be antisense. For construction of the different mutants, the following primers were used: $MDP_{DSS}$ 5′-CGG CGG GAG GTG GAG GCT GGG TGA TGA GTC GTA GCC GTA GCC GTA ATT CGT CCG GCA GG-3′ and $MDP_{DSS}\Delta G$, 5′-GCT GGG TGA TGA GTC GTA GTA ATT CGT CCG GCA GG-3′. Mutations were verified by DNA sequencing. Each mutant was then subcloned back to the wild-type plasmid pEF-MDP by restriction with *Bbs*I, and the region around the mutation was sequenced. The secreted form of MDP has a disrupted GPI signal sequence with the seven most C-terminal amino acids in wild-type MDP replaced with the sequence PASTLP (S. Keynan, A. J. Turner and N. M. Hooper, unpublished).

### Cell growth and expression

COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (heat inactivated), 100 i.u./ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, at 37°C in 5% $CO_2$. The expression vector pEF-BOS (Mizushima and Nagata, 1990) was used to transfect the cells. For transient expression, COS-1 cells were plated in 24-well plates at 33% confluency or in 150 $cm^2$ flasks at approximately $2\times10^6$ cells per flask. After 24 hours of growth, the cells were washed twice with Opti-MEM and transfected (0.2 μg of DNA/well or 5 μg of DNA/flask) using lipofectAmine as cationic lipid (1:10, DNA:lipid). The cells were incubated for 2 hours, and DMEM containing 10% fetal calf serum was added. After 24 hours, the medium was replaced with fresh and the cells incubated for another 24 hours. The medium was then harvested, the cells were washed three times with phosphate-buffered saline (PBS), scraped into 5 ml of PBS and pelleted by centrifugation at 1000 ***g*** for 5 minutes. The cell pellet was resuspended in 4 ml 50 mM Hepes/NaOH, pH 7.5 and disrupted by $N_2$ cavitation at 850 psi for 10 minutes. Membranes were prepared by centrifugation at 1000 ***g*** for 10 minutes at 4°C to remove nuclei, and further centrifugation of the supernatant at 50,000 ***g*** for 1 hour at 4°C using a Beckman L8-55 ultracentrifuge and TLA 100.4 rotor. Membrane pellets were finally resuspended in 0.1 ml 50 mM Hepes/NaOH, pH 7.5.

### PI-PLC incubation, Triton X-114 phase separation and membrane dipeptidase assay

For release of cell-surface proteins with phosphatidylinositol-specific phospholipase C (PI-PLC), cells in 24-well plates were washed twice with PBS 48 hours post-transfection and assayed for MDP activity by the addition of 0.2 ml 3 mM Gly-D-Phe in 0.1 M Tris/HCl, pH 8.0. Cells were again washed with PBS and then incubated with 1 unit *Bacillus thuringiensis* PI-PLC (a gift from Dr M. G. Low, Columbia University, New York) for 1 hour at 37°C. Cells were washed twice with PBS before reassaying for cell-surface MDP activity.

Membranes prepared from COS-1 cells were incubated in the absence or presence of 1 unit/ml *B. thuringiensis* PI-PLC for 1 hour at 37°C and then subjected to temperature-induced phase separation in Triton X-114 (Bordier, 1981). Precondensed Triton X-114 was added to the membrane samples at a final concentration of 1%, and after 5 minutes of incubation at 4°C, the mixture was incubated at 30°C for 3 minutes, before centrifugation at 3000 ***g*** for 3 minutes to separate the upper detergent-poor phase from the lower detergent-rich phase. The two phases were assayed for MDP activity with 1 mM Gly-D-Phe in 0.1 M Tris/HCl, pH 8.0, for 1.5 hours at 37°C. The released D-Phe was separated from the substrate and quantitated by reverse phase HPLC (Hooper et al., 1987).

### Antibody preparation

The polyclonal antibody raised against purified porcine kidney MDP was prepared as described previously (Littlewood et al., 1989). Peptides CRTNYGYS-amide and CAAPSLH-amide, corresponding to amino acids 361-368 and 369-374, respectively, either side of the GPI anchor attachment site in porcine kidney MDP (Fig. 1) (Brewis et al., 1995), were coupled via an N-terminal Cys to keyhole limpet hemocyanin using standard procedures (Baldwin, 1994). New Zealand White rabbits were immunised with the conjugated peptides according to standard protocols (Baldwin, 1994). Peptide (1.0 mg) was injected at intervals of 3-4 weeks, followed by a bleed 10 days after the fourth injection. Serum was stored at −70°C prior to isolation of the IgG fraction by chromatography on Protein G-Superose.

Specificity and sensitivity of antibody production was determined by enzyme-linked immunosorbent assay (Hooper et al., 1991).

### SDS-PAGE and immunoblotting

Samples were mixed with an equal volume of either reducing or non-reducing electrophoresis sample buffer and boiled for 3 minutes. Proteins were resolved by SDS-polyacrylamide gel electrophoresis using a 7-17% acrylamide gradient gel and transferred to Immobilon P poly(vinylidene difluoride) membranes as described previously (Hooper and Turner, 1987). The membranes were blocked by incubation in PBS containing 0.1% (v/v) Tween 20, 5% (w/v) dried milk powder and 2% (w/v) bovine serum albumin overnight at 4°C. All primary and secondary antibody incubations were performed in the same buffer as that used for blocking. Bound antibody was detected using peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence detection method (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK). Protein was quantified using bicinchoninic acid (Smith et al., 1985) in a microtitre plate assay with bovine serum albumin as standard.

### Immunocytochemistry

Cells were grown on clean sterile coverslips in 24-well plates to approximately 30% confluency. Cells were then transfected with 0.2 μg of DNA. Cells were washed 48 hours post-transfection with PBS at 37°C three times before fixing with 4% paraformaldehyde for 20 minutes at room temperature. Cells were washed three times with warm Tris-buffered saline (TBS) to quench free formaldehyde groups and then incubated with the anti-MDP polyclonal antibody diluted 1:200 in TBS containing 0.2% gelatin and 1% goat serum for 2 hours at room temperature. Cells were washed three times in warm TBS prior to incubation with a fluorescein isothiocyanate-conjugated anti-rabbit antibody diluted 1:50 in TBS containing 2% gelatin and 1% goat serum. Cells were washed three times before being mounted on slides, and viewed under a Zeiss microscope.

## RESULTS

### GPI cleavage/attachment site mutants of membrane dipeptidase

To investigate the requirements for GPI anchoring in mammalian cells, the following mutants of the naturally GPI anchored mammalian protein MDP were made (Fig. 1). In the first mutant the entire ω, ω+1 and ω+2 triplet of MDP (Ser-Ala-Ala) was replaced with that present in VSG (Asp-Ser-Ser), generating $MDP_{DSS}$. (Note that the three-letter subscript denotes the residues in the ω, ω+1 and ω+2 positions). Examination of the sequence around the GPI cleavage/attachment site in MDP using the rules for predicting the site of GPI anchor addition in proteins (Udenfriend and Kodukula, 1995b) (see legend to Fig. 1) revealed the presence of possible alternative cleavage/attachment sites upstream of the known site involving Gly366. Potentially this Gly residue could be an alternative ω+2 residue lying in the sequence Asn-Tyr-Gly (probability 0.56) or an alternative ω residue lying in the sequence Gly-Tyr-Ser (probability 0.12). Thus $MDP_{DSS}$ was modified further by deleting Gly366 generating $MDP_{DSS}\Delta G$ in order to remove these alternative GPI cleavage/attachment sites. Both mutants possessed the same hydrophilic spacer and hydrophobic C-terminal sequence as that present in wild-type MDP. The presence of multiple Pro residues (including one at the ω+3 position), and bulky Leu and His residues in the spacer region in wild-type MDP (Fig. 1) should prevent GPI cleavage/attachment occurring within this region, as such residues cannot function in any of the ω, ω+1 or ω+2 positions (Kodukula et al., 1993).

| | 361 ω<br>\| \| Spacer *Hydrophobic domain* | Probability |
|---|---|---|
| Wild type $MDP_{SAA}$ | ..CRTNYGY**SAA**PSLHLPPGS*LLASLVPLLLLSLP* | 1.0 |
| $MDP_{DSS}$ | ..CRTNYGY**DSS**PSLHLPPGS*LLASLVPLLLLSLP* | 0.12 |
| $MDP_{DSS}\Delta G$ | ..CRTNY-Y**DSS**PSLHLPPGS*LLASLVPLLLLSLP* | 0.12 |
| VSG117 | ..*W*ENNACK**DSS**ILVTKK*FALTVVSAAFVALLF* | 0.12 |

**Fig. 1.** Schematic diagram showing the C-terminal sequences in wild-type MDP, the mutant MDPs and wild-type VSG. The sequence of the C terminus of porcine MDP precursor is shown starting with amino acid 361, which is the sole Cys involved in disulphide linking of the homodimer (Keynan et al., 1996). The residues at the cleavage/attachment site (ω, ω+1 and ω+2) are shown in bold. The hydrophilic spacer region is indicated above the sequence, and the C-terminal hydrophobic domain in italics. The predicted probability for the indicated ω residue is based on the ω, ω+2 rule for predicting the site of GPI anchor addition in proteins (Udenfriend and Kodukula, 1995b), e.g. for the cleavage/attachment site Ser-Ala-Ala, the probability of GPI anchorage occurring on the ω residue (Ser) is 1×1=1 (i.e. most probable), whilst for the sequence Asp-Ser-Ser the probability of anchoring occurring on the Asp is 0.4×0.3=0.12 (i.e. much less probable). The C-terminal sequence of *Trypanosoma brucei* VSG117 is shown for comparison.

### Cell-surface expression of the GPI cleavage/attachment site mutants

The mutated proteins were transfected into COS-1 cells, and their cellular location determined. The total cellular activity of both $MDP_{DSS}$ and $MDP_{DSS}\Delta G$ was comparable to that of wild-type MDP (Fig. 2a), indicating that the mutations at the cleavage/attachment site did not alter significantly the level of expression in mammalian cells of MDP. Incorrect processing of a GPI signal sequence usually leads to intracellular retention and degradation of the protein (Delahunty et al., 1993; Field et al., 1994; Moran and Caras, 1992; Moran et al., 1991). In order to determine whether the mutants were being processed in the COS-1 cells, we quantitated the cell-surface MDP activity (Fig. 2b). No significant difference in the amount of MDP activity at the cell-surface was observed between wild-type MDP and either of the mutants. The percentage of the total cell activity at the cell-surface for cells expressing wild-type MDP, $MDP_{DSS}$ or $MDP_{DSS}\Delta G$ was 75.8±4.2%, 82.8±2.6% and 69.7±5.6%, respectively. Confirmation of the cell-surface location of the MDP GPI cleavage/attachment site mutants was obtained by immunofluorescence staining of the cells (Fig. 3). Both $MDP_{DSS}$ and $MDP_{DSS}\Delta G$ gave a similar pattern of cell-surface labelling to wild-type MDP, again indicating that the mutations had not perturbed the transport of MDP to the cell-surface.

### GPI anchoring of the cleavage/attachment site mutants

To confirm that the mutations at and around the GPI cleavage/attachment site were able still to act as viable GPI anchor addition signals in the COS-1 cells, the ability of bacterial PI-PLC to cleave the added GPI anchor was

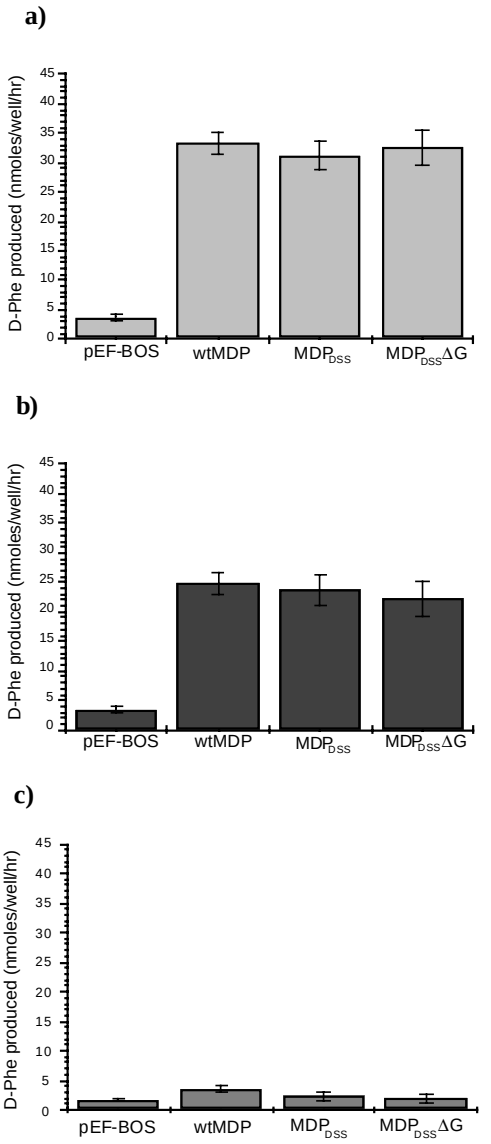


**Fig. 2.** MDP activity of transfected COS-1 cells. Cells in 24-well plates were transfected with 0.2 μg plasmid DNA with either empty vector (pEF-BOS), or vector containing wild-type MDP or the indicated mutants. Cells were washed twice with PBS 48 hours post-transfection and assayed for (a) total cellular MDP activity by the addition of 0.2 ml of 3 mM Gly-D-Phe in 0.1 M Tris/HCl, pH 8.0 containing 1% Triton X-100, as described in Materials and Methods (b) cell-surface MDP activity by the addition of 0.2 ml of 3 mM Gly-D-Phe in 0.1 M Tris/HCl, pH 8.0, or (c) cell-surface activity following incubation of the cells with 1 unit *B. thuringiensis* PI-PLC for 1 hour at 37°C. Results are the mean (± s.e.m.) of five separate experiments.

assessed. Upon incubation of cells expressing wild-type MDP, $MDP_{DSS}$ or $MDP_{DSS}\Delta G$ with PI-PLC, virtually all the MDP cell-surface activity (Fig. 2c) and immunoreactivity (Fig. 3) was released into the medium, with very little remaining attached to the plasma membrane. In case there was an intracellular pool of non-GPI anchored MDP, membranes isolated from cells expressing wild-type MDP or one of the two mutants were incubated with PI-PLC and then subjected to temperature-induced phase separation in Triton

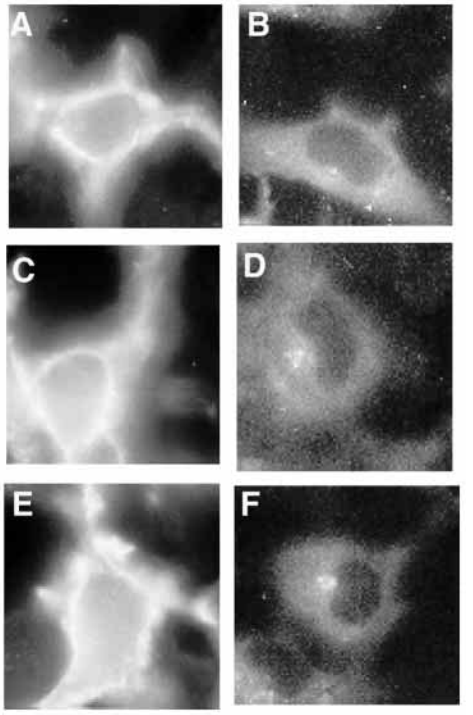


**Fig. 3.** Immunofluorescence staining of transfected COS-1 cells. Cells were grown on coverslips and transfected with 0.2 μg of the indicated DNA. Cells were incubated in the absence (A,C,E) or presence (B,D,F) of bacterial PI-PLC, prior to incubation with the anti-MDP polyclonal antiserum, washed and incubated with a fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. Fluorescence was visualised under a Zeiss microscope. (A,B) Cells transfected with wild-type MDP; (C,D) cells transfected with $MDP_{DSS}$; (E,F) cells transfected with $MDP_{DSS}\Delta G$.

X-114 to separate the uncleaved and cleaved forms (Bordier, 1981; Hooper et al., 1987). In the absence of added PI-PLC both mutants were recovered in the detergent-rich phase (Table 1), as expected for the amphipathic membrane-bound form of MDP (Hooper and Turner, 1989). Following incubation with PI-PLC the majority (>75%) of the MDP activity of both $MDP_{DSS}$ and $MDP_{DSS}\Delta G$ was recovered in the detergent-poor phase (Table 1), with no significant difference as compared to wild-type MDP. Thus both mutants of MDP were being as efficiently GPI anchored in mammalian cells as wild-type MDP, with no evidence for an intracellular pool of unprocessed protein.

**Table 1. PI-PLC cleavage of MDP**

| | Activity in detergent-poor phase (% of total) | | |
|---|---|---|---|
| | Wild-type MDP | $MDP_{DSS}$ | $MDP_{DSS}\Delta G$ |
| –PI–PLC | 10.2±1.9 | 9.4±1.0 | 8.3±1.9 |
| +PI–PLC | 79.8±1.5 | 75.6±4.5 | 76.6±2.9 |

Cells in 150 $cm^2$ flasks were transfected with vector containing wild-type MDP or the indicated mutants. Membranes were prepared from the harvested cells and then incubated in the absence or presence of 1 unit *B. thuringiensis* PI-PLC for 1 hour at 37°C before separation of the intact and cleaved forms of MDP by phase separation in Triton X-114. The activity in the resulting detergent-poor phase is represented as a percentage of the total activity in the detergent-poor and detergent-rich phases.

Results are the means (± s.e.m.) of five separate experiments.

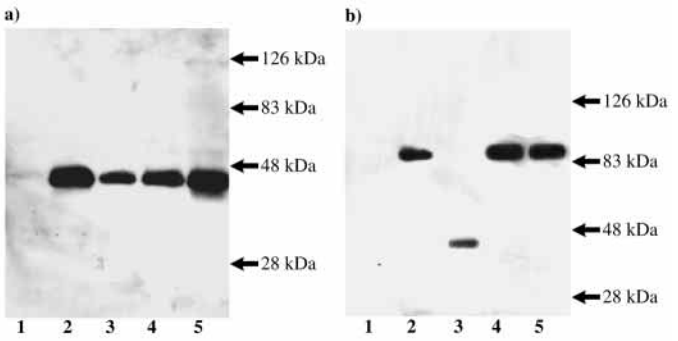


**Fig. 4.** Immunoblot analysis of transfected COS-1 cells. Cells were transfected with either empty vector, or vector containing wild-type MDP or the indicated mutants, then harvested and membranes prepared as described in Materials and Methods. Samples (25 μg protein) were loaded onto 7%-17% polyacrylamide gels and subjected to electrophoresis under (a) reducing or (b) non-reducing conditions. Following transfer to Immobilon P membranes and incubation with anti-MDP antiserum, bound antibody was detected with a peroxidase-conjugated goat anti-rabbit secondary antibody in conjunction with the enhanced-chemiluminescent detection system. Lane 1, membranes from empty vector transfected cells; lane 2, wild-type MDP; lane 3, MDP C361G; lane 4, $MDP_{DSS}$; lane 5, $MDP_{DSS}\Delta G$. The positions of marker proteins are shown.

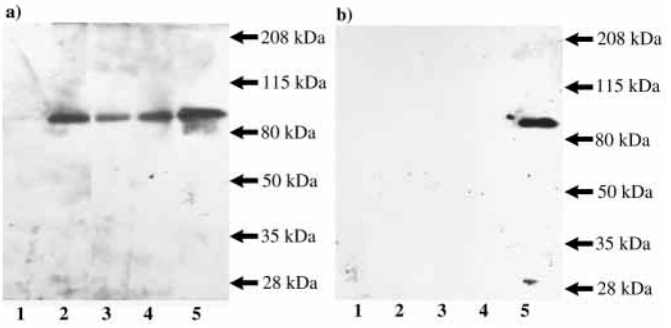


**Fig. 5.** Immunoblot analysis of MDP constructs with antibodies either side of the GPI anchor addition site. Cells were transfected with either empty vector, or vector containing wild-type MDP or the indicated mutants, then harvested and membranes prepared as described in Materials and Methods. Medium was collected from cells expressing the secreted form of MDP, which has a disrupted GPI signal sequence (see Materials and Methods). Samples (25 μg protein) were loaded onto 7%-17% polyacrylamide gels and subjected to electrophoresis under non-reducing conditions. Following transfer to Immobilon P membranes and incubation with either (a) anti-CRTNYGYS antiserum or (b) anti-CAAPSLH antiserum, bound antibody was detected with a peroxidase-conjugated goat anti-rabbit secondary antibody in conjunction with the enhanced-chemiluminescent detection system. Lane 1, membranes from empty vector transfected cells; lane 2, wild-type MDP; lane 3, $MDP_{DSS}$; lane 4, $MDP_{DSS}\Delta G$; lane 5, medium from cells expressing secreted MDP.

### Use of an alternative GPI cleavage/attachment site

There are several reports that upon disruption of the normal GPI cleavage/attachment site, an alternative cleavage/attachment site is used (see e.g. Bucht and Hjalmarsson, 1996; Yan and Ratnam, 1995). In order to determine whether the predicted GPI anchor attachment site had been used in the two mutants of MDP, we analysed the expressed proteins by SDS-PAGE and western blotting. Under reducing conditions $MDP_{DSS}$ and $MDP_{DSS}\Delta G$ migrated with the same apparent molecular mass as the wild-type N-glycosylated MDP of 47 kDa (Fig. 4a). On this gel system a difference of as little as 2 kDa at this molecular mass is clearly visible (Littlewood et al., 1989), indicating that the mutants are not significantly different in size from wild-type MDP and that an alternative GPI cleavage/attachment site distant from the expected one had not been used. Further evidence for the use of the expected GPI cleavage/attachment site was obtained by analysis of the proteins on SDS-PAGE under non-reducing conditions. MDP is a disulphide-linked dimer with Cys361, at position ω-7 (Fig. 1), the only residue involved in the interchain dimerisation (Keynan et al., 1996). Mutation of Cys361 to Gly causes the protein to migrate as a monomer under non-reducing conditions (Fig. 4b, lane 3). However, under non-reducing conditions both $MDP_{DSS}$ and $MDP_{DSS}\Delta G$ migrated as disulphide-linked dimers (Fig. 4b, lanes 4 and 5), indicating that the GPI anchor was not added N-terminal to Cys361.

Additional evidence for attachment of the GPI anchor at the expected site in both of the MDP mutants came from the use of site-specific antibodies that recognise sequences either side of the native ω residue, Ser368, in wild-type porcine MDP (Brewis et al., 1995). The anti-CRTNYGYS peptide antibody recognises the region N-terminal to Ser368 (Fig. 1). This sequence does not appear elsewhere in porcine MDP. Either one or both of the Tyr residues in this peptide appear to be critical for recognition by this antibody, as modification of them with $^{125}I$ abolishes recognition (S. P. Heywood and N. M. Hooper, unpublished). On western blot analysis of membranes from COS-1 cells expressing wild-type MDP or one of the two MDP mutants, the anti-CRTNYGYS antibody recognised a polypeptide of 94 kDa in membranes from cells expressing either $MDP_{DSS}$ or $MDP_{DSS}\Delta G$ (Fig. 5a), indicating that the GPI anchor was not added N-terminal to the expected ω site.

The anti-CAAPSLH peptide antibody recognises the residues ω+1 to ω+6 immediately C-terminal to the native ω site in porcine MDP (Fig. 1). As expected, this antibody failed to recognise any protein on western blot analysis of membranes from cells expressing wild-type MDP (Fig. 5b, lane 2) as this epitope is removed on addition of the GPI anchor. However, the anti-CAAPSLH antibody did recognise a secreted form of MDP, which has a disrupted GPI anchor addition signal and retains this sequence, and which is efficiently secreted from COS cells (I. J. White and N. M. Hooper, unpublished) (Fig. 5b, lane 5). The anti-CAAPSLH antibody failed to recognise either $MDP_{DSS}$ or $MDP_{DSS}\Delta G$ (Fig. 5b, lanes 3 and 4), indicating that neither of them had been anchored C-terminal to the expected ω site, and that there was not a significant intracellular pool of unprocessed protein retaining this sequence.

## DISCUSSION

The vast majority of mammalian GPI anchored proteins in which the cleavage/attachment site has been unequivocally identified have either Ala or Gly at the ω+2 position, whereas virtually all the protozoal GPI anchored proteins have Ser at this position (see Bucht and Hjalmarsson, 1996; Furukawa et al., 1997) for recent lists of GPI anchored proteins and their

cleavage/attachment sites). Although a fusion protein of human growth hormone and the C-terminal sequence of trypanosome VSG were inefficiently GPI-anchored in COS cells, conversion of the VSG GPI cleavage/attachment site from Asp-Ser-Ser to Asp-Ser-Ala or substitution with the sequence Ser-Gly-Thr from mammalian decay accelerating factor led to efficient GPI anchoring of the resulting fusion protein (Moran and Caras, 1994). This led to the hypothesis that the binding pocket in the mammalian transamidase is only large enough to accommodate two Ser residues in the ω and ω+2 positions; if the ω position is occupied by the slightly larger Asp, then to compensate, the ω+2 residue must be smaller, allowing only Ala or Gly. In contrast the trypanosome transamidase has a larger binding site, which can accommodate Asp at the ω position and Ser at the ω+2 position (Moran and Caras, 1994).

We examined this hypothesis from the opposite direction, taking the naturally GPI anchored mammalian protein, MDP, and mutating its GPI cleavage/attachment site from Ser-Ala-Ala to that present in VSG, Asp-Ser-Ser, reasoning that if the hypothesis is correct the resulting mutant would be inefficiently processed in COS-1 cells. An additional mutant was created in which possible alternative GPI cleavage/attachment sites were abolished by deletion of Gly366. Both of the resulting mutants $MDP_{DSS}$ and $MDP_{DSS}\Delta G$ were found to be equally efficiently GPI anchored, N-glycosylated and targeted to the plasma membrane in an enzymically active form as the wild-type protein. Using site-specific antibodies recognising epitopes either side of the expected cleavage/attachment site we could obtain no evidence for use of an alternative GPI cleavage/attachment site in either mutant. Thus our data indicate that the mammalian transamidase can accommodate two relatively large residues (Asp and Ser) in the ω and ω+2 sites, respectively. Although it was reported that neither the length of the hydrophilic spacer region nor the properties of the C-terminal hydrophobic domain were responsible for the inefficient GPI anchoring of VSG in mammalian cells (Moran and Caras, 1994), residues more distant from the cleavage/attachment site may influence the efficiency of GPI anchoring, possibly in a protein-specific manner (Aceto et al., 1999; Eisenhaber et al., 1998). Certainly our data indicate that there is not such a difference in requirements for GPI anchoring between mammalian and protozoal cells as previously hypothesised.

Using the ω, ω+2 rule of Udenfriend and Kodukula (1995b) both of the mutants $MDP_{DSS}$ and $MDP_{DSS}\Delta G$ have predicted GPI anchoring probabilities of 0.12 (see legend to Fig. 1), suggesting that they should be inefficiently processed. However, we were unable to detect significant differences in the efficiency of GPI anchoring of either of these mutants as compared to wild-type MDP, which has a predicted GPI anchoring probability of 1.0 (i.e. the highest probability). These results would suggest that the experimentally determined requirements for GPI anchoring of one protein (placental alkaline phosphatase) do not necessarily hold true for another protein, and that caution must be exercised when using such predictions to determine potential GPI cleavage/attachment sites in another protein. In a recent report the sequence requirements for GPI cleavage/attachment were investigated in a chimera consisting of the ectodomain of mini-placental alkaline phosphatase and the C-terminal portion of the urokinase receptor (Aceto et al., 1999). The requirements for processing of the chimeric protein were markedly different to those previously reported for mini-placental alkaline phosphatase, leading to a similar conclusion as the present study, that the requirements for GPI anchoring are more complex than previously proposed.

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