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The carboxyl terminus of *Dictyostelium discoideum* protein 1I encodes a functional glycosyl-phosphatidylinositol signal sequence[1]

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Abstract

The 1I gene is expressed in the prespore cells of culminating *Dictyostelium discoideum*. The open reading frame of 1I cDNA encodes a protein of 155 amino acids with hydrophobic segments at both its NH_2- and COOH-termini that are indicative of a glycosyl-phosphatidylinositol (GPI)-anchored protein. A hexaHis-tagged form of 1I expressed in *D. discoideum* cells appeared on Western blot analysis as a doublet of 27 and 24 kDa, with a minor polypeptide of 22 kDa. None of the polypeptides were released from the cell surface with bacterial phosphatidylinositol-specific phospholipase C, although all three were released upon nitrous acid treatment, indicating the presence of a phospholipase-resistant GPI anchor. Further evidence for the C-terminal sequence of 1I acting as a GPI attachment signal was obtained by replacing the GPI anchor signal sequence of porcine membrane dipeptidase with that from 1I. Two constructs of dipeptidase with the 1I GPI signal sequence were constructed, one of which included an additional six amino acids in the hydrophilic spacer. Both of the resultant constructs were targeted to the surface of COS cells and were GPI-anchored as shown by digestion with phospholipase C, indicating that the *Dictyostelium* GPI signal sequence is functional in mammalian cells. Site-specific antibodies recognising epitopes either side of the expected GPI anchor attachment site were used to determine the site of GPI anchor attachment in the constructs. These parallel approaches show that the C-terminal signal sequence of 1I can direct the addition of a GPI anchor.

Keywords: Cellular slime mold; Glycosyl-phosphatidylinositol; Membrane dipeptidase; Phospholipase C; *Dictyostelium discoideum*

1. Introduction

Many intercellular communication processes rely on proteins of the plasma membrane for signal transduction. Although many integral membrane proteins are anchored by hydrophobic transmembrane domains, others are modified by the posttranslational addition of a glycosyl-phosphatidylinositol (GPI) anchor. These complex glycan structures containing the core sequence ethanolamine-PO_4-6Manα1-2Manα1-6Manα1-4Glc$NH_2\alpha$1-6-*myo*-inositol-1-PO_4-lipid are attached to the C-terminus of proteins, anchoring

Abbreviations: csA, contact site A; ER, endoplasmic reticulum; GPI, glycosyl-phosphatidylinositol; MDP, membrane dipeptidase; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; PsA, prespore-specific antigen; PVDF, poly(vinylidene difluoride); SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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them to the outer leaflet of the lipid bilayer, and have been described in protozoa, yeast, invertebrates, vertebrates and cellular slime molds [1]. In cellular slime molds the presence of a GPI anchor has been demonstrated experimentally for the *Dictyostelium discoideum* contact site A (csA) and prespore-specific antigen (PsA) glycoproteins [2,3] and the *Polysphondylium pallidum* gp64 [4], and is predicted for the *D. discoideum* gp138 [5].

A protein destined to be GPI anchored is translated with an N-terminal signal peptide that directs the growing polypeptide to the endoplasmic reticulum (ER) for co-translational insertion into the membrane, and which is then removed in the ER lumen by signal peptidase. 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 [6,7]. 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 in mammalian proteins [8]. 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 the 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 [9,10], decay accelerating factor [11] and yeast Gas1 protein [12] 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 for predicting the site of GPI anchor addition in proteins have been devised based on these experimental observations [8,13].

We report here that *D. discoideum* protein 1I, which has been shown previously to be expressed in a cell type-specific manner during asexual development [14], contains a C-terminal signal sequence indicative of a GPI-anchored protein, and present two parallel approaches to demonstrate that this signal sequence is functional. First, nitrous acid treatment, which cleaves the glucosamine-inositol bond in GPI anchors, released a hexaHis-tagged form of the 1I protein from *D. discoideum* membranes. Second, replacement of the GPI signal sequence of porcine membrane dipeptidase (MDP; EC 3.4.13.19) [15,16] with that of the putative C-terminal GPI signal sequence of 1I resulted in a protein that was expressed at the surface of COS cells in a GPI-anchored form. The data show that the 1I GPI anchor sequence is functional and, in addition, that a *D. discoideum* GPI anchor signal sequence can function in mammalian cells.

2. Materials and methods

2.1. Sequence analysis of the 1I family of cDNA clones

Escherichia coli cells containing the 1I cDNA clone in pAT153 were grown in Luria-Bertani medium containing 15 μg/ml tetracycline, and plasmids were recovered from the cells using the Qiagen Midi kit following the manufacturer's recommended protocols. Sequencing data were obtained using an ABI-PRISM automated sequencer with the primers pAT-For (5′-CGC CAG TTA ATA GTT TGC-3′) and pAT-Rev (5′-GAA GCC ATA CCA AAC GAC-3′) which flank the *Pst*I site of pAT153. Sequencing data obtained were used to design two internal primers, 1I-Ups (5′-TGT CCA GCT TTG GTA AAT A-3′) and 1I-Downs (5′-AAA TAT ACA GTG GTG GTT CC-3′) which were then used in combination with the pAT153 sequencing primers to detect other 1I family clones containing extensions of cDNA sequence and subsequently for the sequencing of these clones. General sequence manipulations were performed using the Wisconsin package, Version 8.1 (Genetics Computer Group, August 1995). DNA and protein searches of the major databases were carried out using BLAST, PSI-BLAST and FASTA programmes.

2.2. Cloning of the 1I/HexaHis construct

The 1I/HexaHis construct was generated using the megaprimer PCR strategy [17]. A megaprimer was synthesised using HexaHis (5′-CAA TAT GCT CAA GTA ACT CAT CAC CAT CAC CAT CAC

GAA ACT CCA GCT GGT AAC-3′) and Pdicty (5′-GCG CGC TCT AGA TGG ACC AGC TGG TGG CTC-3′) primers, and DNA prepared from clone 7B as a template. The reverse strand of this PCR product was then utilised as the megaprimer in a second PCR reaction against HisStart (5′-GCG CGC TCT AGA AGA TTC ATT TCA ATT TTT-3′) using DNA prepared from clone 7I as a template. This PCR product was digested with *Xba*I and cloned into PVEII vector (a generous gift of Prof. W. Nellen, Universität Gesamthochschule, Kassel) that had been linearised with the same enzyme before alkaline phosphatase treatment.

2.3. Generation of membrane dipeptidase constructs and expression in COS cells

The MDP/1I fusion constructs MDP-GPI23 and MDP-GPI29 were also generated using the megaprimer PCR strategy [17]. MDP-GPI23 megaprimer was synthesised using PFuse (5′-CGG ACG AAT TAC GGC TAC AAC TCT GCT GAT AAA GTA GCC GTT GGT-3′) and PEnd (5′-GCG CGC TCT AGA TTA AAG TGC TAA GAG TGA-3′) primers, and DNA prepared from clone 7B as a template. The reverse strand of this PCR product was then utilised as the megaprimer in a second PCR reaction against pStart (5′-GCG CGC TCT AGA CAG ACG TGA GGA GCG GCT-3′) using DNA prepared from MDP in pEF-BOS as a template [18]. This PCR product was digested with *Xba*I and cloned into pEF-BOS vector also cut with this enzyme before treatment with alkaline phosphatase to generate the MDP-GPI23 construct. The MDP-GPI29 construct was synthesised by the same method, but using PFuselink primer (5′-CGG ACG AAT TAC GGC TAC AAC TCT GCT CCC AGC CTC CAC CTC CCG GAT AAA GTA GCC GTT GGT-3′) in place of PFuse in the first PCR reaction. The resulting constructs were transiently expressed in COS cells as described previously [19].

2.4. Expression in Dictyostelium

Vegetative *Dictyostelium* cells were transformed essentially as described by Nellen et al. [20] but with the modifications of Early and Williams [21] for both the 1I/HexaHis construct and empty PVEII vector. Transformed cells were selected in the presence of 20 μg/ml G418, and 1 mM folate was included to repress recombinant protein expression. Pooled populations of stable transformants were then grown axenically in HL-5 medium containing both G418 and folate. Cells were washed in folate-free medium and grown for 24 h in fresh folate-free medium before harvesting to derepress expression from PVEII. Development was induced on nitrocellulose filters as described previously [22]. Cells were lysed by three cycles of freeze/thaw, and total protein extracts made by boiling the lysate in electrophoresis sample buffer. A total cell membrane fraction was prepared by centrifugation of the cell lysate at $100\,000\times g$ for 1 h before resuspension in 50 mM HEPES/NaOH (pH 7.5).

2.5. Enzymic deglycosylation

Cell extracts were treated with peptide *N*-glycosidase F (Oxford Glycosciences) at 37°C overnight in accordance with the manufacturer's recommended protocol. Porcine MDP was included as a positive control. Cell extracts were treated with neuraminidase (Sigma) and *O*-glycanase (Oxford Glycosciences) at 25°C overnight in accordance with the manufacturer's recommended protocol. Human testicular angiotensin-converting enzyme was included as a positive control.

2.6. Phospholipase C treatment

Derepressed Ax2 cells were washed in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl before resuspension of aliquots of 10^7 cells in 0.5 ml of the same buffer. Cells were shaken at 200 rpm at 22°C for 2 h with or without the addition of 1 unit of *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) (a kind gift of Dr. M.G. Low, Columbia University, New York), before the cells were pelleted by centrifugation and cell pellets and supernatants frozen separately on dry ice. Aliquots (10 μl) of total cell membrane preparations in 50 mM HEPES/NaOH (pH 7.5) were incubated at 22°C for 12 h with or without the addition of 0.1 units *B. thuringiensis* PI-PLC before the membranes were pelleted by centrifugation at $100\,000\times g$ for 1 h.

For release of proteins from the surface of COS

cells with PI-PLC, cells in 24-well plates were washed twice with phosphate-buffered saline (PBS) 48 h post transfection and assayed for MDP activity by the addition of 0.2 ml of 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 *B. thuringiensis* PI-PLC for 1 h at 37°C. Cells were washed twice with PBS before reassaying for cell surface MDP activity. The released D-Phe was separated from the substrate and quantitated by reverse phase HPLC [23].

2.7. Nitrous acid treatment

Cell membranes were resuspended in 0.25 M sodium acetate (pH 4.0) containing 0.25 M freshly dissolved $NaNO_2$ and incubated for 4 h at 22°C. Control samples were treated similarly but with the inclusion of 0.25 M NaCl in place of the $NaNO_2$. After centrifugation at $100\,000 \times g$ for 1 h, the pelleted membranes were resuspended in 1 vol. of 0.25 M sodium acetate (pH 4.0), and 1.5 vols. of 1 M Tris-HCl (pH 7.6) were added to both the pellet and supernatant fractions to neutralise the samples.

2.8. SDS-PAGE and Western blot analysis

Samples were mixed with an equal volume of either reducing or non-reducing electrophoresis sample buffer (0.14 M Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, ±0.1% dithiothreitol) and boiled for 3 min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using either a 7–17% polyacrylamide gradient gel or a 15% polyacrylamide gel, and transferred to Immobilon P poly(vinylidene difluoride) (PVDF) membranes as described previously [24]. HexaHis-tagged protein was detected using Qiagen anti-TetraHis and anti-PentaHis primary antibodies and following the manufacturer's recommended protocols. For the detection of 1I/MDP fusion proteins, membranes were first 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. Primary and secondary antibody incubations were performed in the same buffer as that used for blocking. The polyclonal antibody raised against purified porcine kidney MDP was prepared as described previously [25]. The antibodies raised against the synthetic peptides CRTNYGYS-amide and CAAPSLH-amide were prepared and characterised as described previously [19]. 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 [26] in a microtitre plate assay with bovine serum albumin as standard.

3. Results

3.1. The coding sequence of the D. discoideum 1I protein predicts a GPI-anchored protein

The cloning of the prespore-specific 1I cDNA family has been described previously [14]. A composite of sequence data spanning 634 nucleotides was generated from the overlapping clones 7I, 1I and 7B (Fig. 1a). The protein encoded by the longest open reading frame is 155 amino acids in length and possesses two signal sequences indicative of a GPI-anchored protein. A hydrophobic signal sequence lies at the N-terminus of the protein and a hydrophobic peptide of some 18 amino acids lies at the very C-terminus of the nascent chain (Fig. 1b). When the N-terminal signal sequence was analysed using the weight-matrix method of von Heijne [27], a probable cleavage site (score of 10.7) was generated at position 20, which would place Phe at the N-terminus of the mature protein. Putative sites of GPI anchor attachment at the C-terminus were analysed by calculating the products of experimentally derived probabilities of anchor attachment for various amino acid substituents at the ω and ω+2 positions [8]. The data (not shown) indicate that Asn133, Ser134 and Ala135 are likely to be at the ω, ω+1 and ω+2 sites, respectively. The potential for GPI anchoring of the 1I protein was also assessed using the 'big-Π' prediction tool available at http://mendel.imp.univie.ac.at/gpi/gpi_server.html [13]. Both the metazoan and protozoan parameterised algorithms predicted that the 1I protein would be GPI-anchored, and both algorithms calculated that Asn133 would constitute the ω site (false positive probabilities calculated as 5.9e-3 and 7.5e-3, respectively).

a)

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1   CGTTTTAAAAAAAAAAAAATAAATATATAAATATATTTACCATTACATTTATTTATTAAT
                                                  M  R  F  I  S   5
61  TTCTTAAAATAATAATAATAATAACAATAACAATTTCAATAGTAAAATGAGATTCATTTC
    I  F  L  I  I  V  A  L  C  V  S  S  S  W  A  F  N  F  T  D  25
121 AATTTTTTTAATAATCGTTGCATTGTGTGTTTCATCAAGTTGGGCATTTAATTTTACTGA
    Q  P  N  S  F  R  I  S  G  T  G  C  G  S  G  T  T  T  V  Y  45
181 CCAACCAAATTCATTTAGAATCTCAGGTACAGGATGTGGTTCTGGAACCACCACTGTATA
    F  S  T  D  G  R  C  N  S  A  C  G  G  S  I  R  I  K  G  E  65
241 TTTTTCAACTGATGGTAGATGTAATTCTGCTTGTGGTGGAAGTATTAGAATAAAAGGTGA
    G  N  N  V  P  N  Q  Q  F  T  L  N  D  Y  S  K  N  V  T  N  85
301 AGGAAATAATGTTCCAAACCAACAGTTTACCCTTAATGATTATAGTAAAAATGTAACAAA
    C  S  G  T  S  N  V  A  S  F  R  C  P  A  L  V  N  T  T  S 105
361 TTGTAGTGGTACCTCAAATGTTGCAAGTTTCAGATGTCCAGCTTTGGTAAATACTACTAG
    P  T  F  T  V  N  V  G  N  S  A  Y  H  V  T  C  Q  Y  A  Q 125
421 TCCAACCTTCACTGTTAATGTAGGAAATTCTGCTTACCACGTTACTTGTCAATATGCTCA
    V  T  •  E  T  P  A  G  N  S  A  D  K  V  A  V  G  I  A  I  I 145
481 AGTAACTGAAACTCCAGCTGGTAACTCTGCTGATAAAGTAGCCGTTGGTATTGCTATTAT
    F  G  A  L  I  S  L  L  A  L  *                              155
541 CTTTGGTGCTTTAATCTCACTCTTAGCACTTTAAAAAATCAACAACAAGGGAAAAAAAAA

601 CAAAAAAAAAAAAAAAAAAAAAAATTTTATAAAT
```

b)

Fig. 1. Composite of sequence data from the 7I, 1I and 7B clones; translation and hydrophilicity of the longest open reading frame. (a) The predicted polypeptide sequence of the longest open reading frame is shown above the nucleotide sequence with amino acids being numbered on the right and nucleotides on the left. The translation stop codon is marked by an asterisk. The N-terminal signal sequence is underscored with a dashed line and the four consensus sites for N-linked glycosylation are double underlined. The predicted ω residue at the cleavage/attachment site is overlined. The hydrophilic spacer region, including the ω+1 and ω+2 residues, is underlined, and the C-terminal hydrophobic domain is italicised. The point at which the hexahistidine tag was incorporated is marked by the symbol •. (b) Hydrophobicity of the 1I open reading frame, calculated by the Kyte-Doolittle algorithm within the Wisconsin (GCG) package.

3.2. *Evidence for a GPI anchor on the hexaHis-tagged 1I protein in D. discoideum*

To investigate the predicted GPI anchoring of the 1I protein in vivo, a hexaHis-tagged form of the protein comprising the entire open reading frame of 1I was constructed in the vector PVEII and expressed in *D. discoideum*. As both N- and C-termini

are lost during the processing of GPI-anchored proteins, the hexaHis tag was incorporated internally between residues Thr127 and Glu128, just upstream from the putative GPI anchor attachment site, Asn133 (Fig. 1a). Two extra residues (Ser and Arg) were incorporated into the N-terminal signal sequence after the initiating Met as a result of the sequence of the multiple cloning site in PVEII. However, analysis of this protein sequence [27] predicted that this signal sequence would be cleaved at the same site as that of the endogenous protein. Western blotting showed that the 1I/HexaHis protein was expressed by vegetative Ax2 cells as a strong doublet at 24 and 27 kDa and a third, weaker band at 22 kDa, and was not secreted (Fig. 2). Cells expressing the 1I/HexaHis protein developed with normal timing and normal morphology (data not shown).

Although cleavage by bacterial PI-PLC is a common diagnostic test for a GPI anchor on a protein, it is notable that neither of the *D. discoideum* proteins PsA or csA are cleaved by exogenous bacterial PI-PLC from either the surface of intact cells or isolated membranes [28,29]. In agreement with this, treatment of intact Ax2 cells with *B. thuringiensis* PI-PLC for up to 2 h, or the treatment of *D. discoideum* cell membranes in the absence or presence of 1% Triton X-114 for 12 h, did not result in release of the 1I/HexaHis protein into the medium (data not shown). Treatment with nitrous acid (which deaminates the glucosamine residue of the GPI anchor and thus releases the protein from the lipids) has been used as an alternative diagnostic test for GPI anchors [29,30]. Treatment of *D. discoideum* cell membranes with nitrous acid caused partial release of 1I/HexaHis protein (Fig. 3, lane 8), indicating the presence of a GPI anchor. This effect was specific for the nitrous acid, as the control treatment at pH 4 but with NaCl replacing the $NaNO_2$ did not cause release of the 1I protein into the soluble fraction (Fig. 3, lane 6).

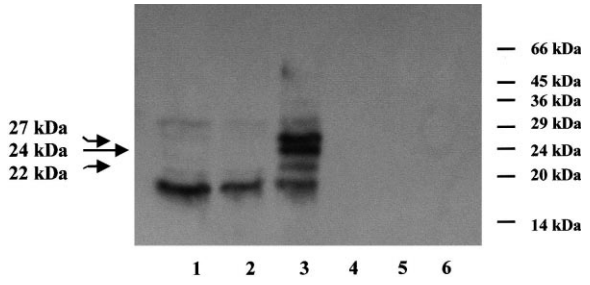

Fig. 2. Expression of 1I/HexaHis protein by Ax2 cells. Ax2 cells were stably transformed with either empty vector (PVEII) or vector containing the 1I/HexaHis construct. Protein expression was derepressed, and cells were harvested as described in Section 2. Samples (approx. 10–20 μg protein) were loaded onto a 15% polyacrylamide gel and subjected to electrophoresis under reducing conditions. Following transfer to PVDF membranes and incubation with anti-His-tag antibodies, bound antibody was detected with a peroxidase conjugated rabbit anti-mouse secondary antibody in conjunction with the enhanced-chemiluminescent detection system. The figure shows protein from (lane 1) untransformed cells, (lane 2) cells transformed with empty vector, (lane 3) cells transformed with 1I/HexaHis construct, (lane 4) supernatant from untransformed cells, (lane 5) supernatant from cells transformed with empty vector, (lane 6) supernatant from cells transformed with 1I/HexaHis construct.

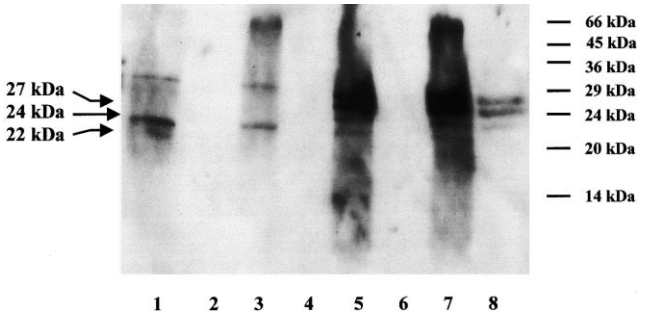

Fig. 3. Effect of nitrous acid treatment on membrane anchoring of 1I/HexaHis protein. Cell membranes were prepared from untransformed Ax2 cells and cells stably transformed with the 1I/HexaHis construct, as described in Section 2. Membranes were treated with either nitrous acid or sodium chloride (as a negative control) for 4 h, before centrifugation to separate membrane bound and released fractions. Membrane bound fractions were resuspended in a volume of sodium acetate equal to that of the supernatant fraction. Protein samples were then prepared as described in Section 2, and equal sample volumes loaded onto a 15% polyacrylamide gel and subjected to electrophoresis under reducing conditions. Following transfer to PVDF membranes and incubation with anti-His-tag antibodies, bound antibody was detected with a peroxidase conjugated rabbit anti-mouse secondary antibody in conjunction with the enhanced-chemiluminescent detection system. The figure shows membranes from (lanes 1–4) untransformed cells, and (lanes 5–8) cells stably transformed with the 1I/HexaHis construct. Lanes: 1 and 5, control treated membrane bound fractions; 2 and 6, control treated released fractions; 3 and 7, nitrous acid treated membrane bound fractions; 4 and 8, nitrous acid treated released fractions.

3.3. MDP/1I fusion proteins are GPI-anchored in COS cells

Porcine MDP is a 47 kDa GPI-anchored ectoenzyme [23] in which the GPI anchor is attached to Ser368 (Fig. 4) [16]. The C-terminal signal sequence in porcine MDP was replaced with that from the 1I protein, generating MDP-GPI23, and another construct MDP-GPI29 was generated which incorporates an additional six amino acids (identical to those in porcine MDP) after the ω+2 residue of the 1I protein sequence (Fig. 4). Wild type MDP and the two constructs MDP-GPI23 and MDP-GPI29 were transfected into COS-1 cells. The total cellular activities of MDP-GPI23 and MDP-GPI29 were identical, and corresponded to 83% of that of the wild type MDP (Fig. 5a), indicating that the *D. discoideum* GPI addition sequence does not alter significantly the level of expression in mammalian cells of MDP. Although incorrect processing of a GPI signal sequence leads to intracellular retention and degradation of the protein [31], the amount of MDP activity

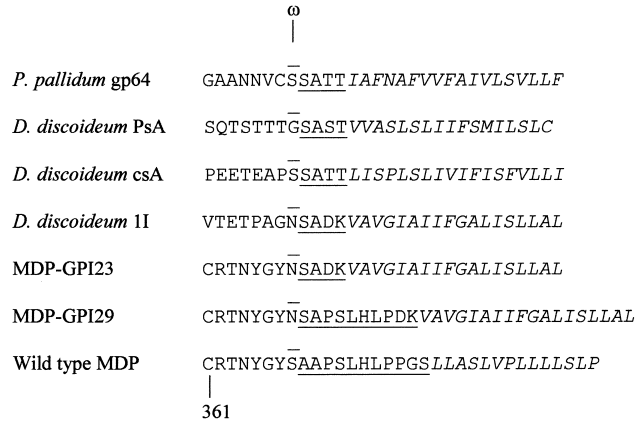

Fig. 4. The C-terminal sequences of *P. pallidum* gp64, *D. discoideum* PsA, csA and 1I, the 1I/MDP fusion proteins and porcine MDP. The sequences of the C-termini of *P. pallidum* gp64, *D. discoideum* PsA, csA and 1I, the 1I/MDP fusion proteins and wild type porcine MDP are shown. The known ω residues at the cleavage/attachment sites of gp64 [4], PsA [28] and MDP [16] are overlined, as are the predicted ω residues of csA [49], 1I, MDP-GPI23 and MDP-GPI29. The hydrophilic spacer regions, including the ω+1 and ω+2 residues, are underlined, and the C-terminal hydrophobic domains are italicised. The sequences of the C-termini of MDP, MDP-GPI23 and MDP-GPI29 are shown starting with amino acid 361, which is the only Cys involved in disulphide linking of the homodimer [32].

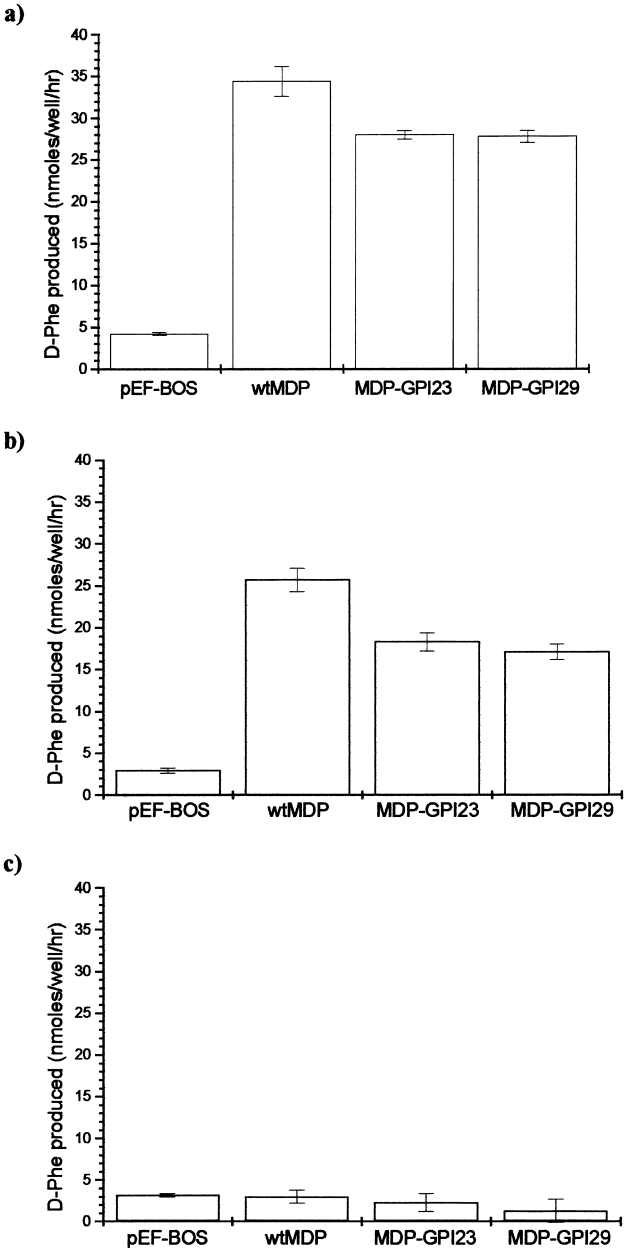

Fig. 5. MDP activity of transfected COS-1 cells. COS cells in 24-well plates were transfected with 0.2 μg plasmid DNA of either empty vector (pEF-BOS), or vector containing wild type MDP or the indicated constructs. Cells were washed twice with PBS 48 h post transfection, and assayed for (a) total cellular MDP activity by the addition of 0.2 ml 3 mM Gly-D-Phe in 0.1 M Tris-HCl (pH 8.0) containing 1% Triton X-100, (b) cell surface MDP activity by the addition of 0.2 ml 3 mM Gly-D-Phe in 0.1 M Tris-HCl (pH 8.0), (c) cell surface activity following incubation of the cells with 1 unit *B. thuringiensis* PI-PLC for 1 h at 37°C. Results shown are the mean (±S.E.M.) of three separate experiments.

at the surface of the COS-1 cells transfected with MDP-GPI23 and MDP-GPI29 were virtually identical, and corresponded to 67% of that of wild type MDP (Fig. 5b). The proportion of the total cellular activity at the surface of the cells transfected with wild type MDP, MDP-GPI23 and MDP-GPI29 were similar (75%, 65% and 61%, respectively). To confirm that the *D. discoideum* sequences were able to act as GPI anchor addition signals in COS-1 cells, the ability of bacterial PI-PLC to cleave the added GPI anchor was assessed. Upon incubation of cells expressing wild type MDP, MDP-GPI23 or MDP-GPI29 with PI-PLC, virtually all ($>89\%$) of the cell surface MDP activity was released into the medium, with very little remaining attached to the plasma membrane (Fig. 5c). Thus, it would appear that the *D. discoideum* GPI anchor signal, with or without the extra length in the hydrophilic spacer region, can direct addition of a GPI anchor in mammalian cells.

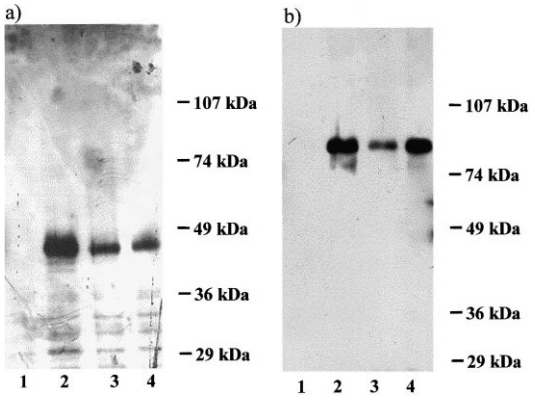

Fig. 6. Expression of MDP constructs in COS-1 cells. COS cells were transfected with either empty vector, or vector containing wild type MDP, MDP-GPI23 or MDP-GPI29. Cells were harvested and membranes prepared as described in Section 2. Samples (25 μg protein) were loaded onto 7–17% polyacrylamide gels and subjected to electrophoresis under (a) reducing or (b) non-reducing conditions. Following transfer to PVDF 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. The figure shows analysed membranes from cells transfected with (lane 1) empty vector, (lane 2) wild type MDP, (lane 3) MDP-GPI23, and (lane 4) MDP-GPI29.

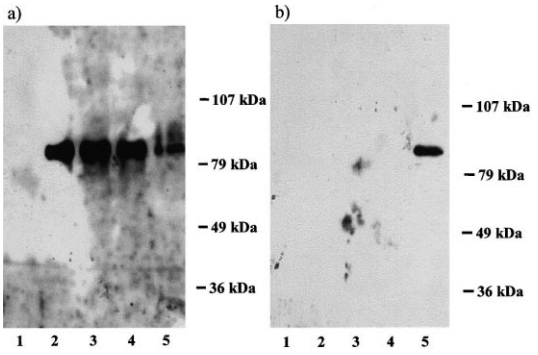

Fig. 7. Analysis of the GPI cleavage/attachment site in the MDP constructs. COS cells were transfected with either empty vector, or vector containing wild type MDP, MDP-GPI23 or MDP-GPI29. Cells were harvested and membranes prepared as described in Section 2. Samples (25 μg protein) were loaded onto 7–17% polyacrylamide gels and subjected to electrophoresis under non-reducing conditions. Following transfer to PVDF membranes and incubation with (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. The figure shows analysed membranes from cells transfected with (lane 1) empty vector, (lane 2) wild type MDP, (lane 3) MDP-GPI23, and (lane 4) MDP-GPI29. Lane 5, concentrated medium from cells transfected with a secreted form of MDP that retains the CAAPSLH epitope [19].

3.4. Determination of the site of GPI attachment in the MDP/1I constructs

On SDS-PAGE under reducing conditions MDP-GPI23 and MDP-GPI29 migrated with the same apparent molecular mass as wild type MDP of 47 kDa (Fig. 6a). On these gel systems a difference of 2 kDa is clearly visible [25], indicating that the constructs are not significantly different in size from wild type MDP and that an alternative GPI attachment site distant from the expected one had not been used. Further evidence for the use of the expected GPI anchor attachment site was obtained by analysis of the proteins on SDS-PAGE under non-reducing conditions. MDP is a disulphide-linked dimer with Cys361 the only residue involved in the interchain dimerisation (Fig. 4) [32]. Under non-reducing conditions both MDP-GPI23 and MDP-GPI29 migrated as disulphide-linked dimers (Fig. 6b), indicating that neither construct had the GPI anchor added N-terminal to Cys361.

Additional evidence for attachment of the GPI anchor at the expected site came from the use of site-specific antibodies that recognise the sequences either side of the native ω residue, Ser368, in porcine MDP. The anti-CRTNYGYS peptide antibody recognises the region N-terminal to Ser368 (Fig. 4). The two Tyr residues in this peptide appear to be critical for recognition by this antibody as modification with ^{125}I abolishes recognition (Heywood and Hooper, unpublished). On Western blot analysis of membranes from COS-1 cells expressing wild type MDP or one of the MDP constructs, the anti-CRTNYGYS antibody recognised a polypeptide of 94 kDa in all cases (Fig. 7a), 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. 4). As expected, this antibody failed to recognise any protein on Western blot analysis of membranes from cells expressing either wild type MDP in which this epitope is removed on addition of the GPI anchor or MDP-GPI23 which does not contain this epitope (Fig. 7b). However, the anti-CAAPSLH antibody did recognise a secreted form of MDP which has a disrupted GPI anchor addition signal and retains this sequence (Fig. 7b) [19]. The anti-CAAPSLH antibody failed to recognise MDP-GPI29 (Fig. 7b), indicating that it had not been anchored C-terminal to the expected ω site, and that there was not a significant intracellular pool of unprocessed protein retaining this sequence.

4. Discussion

The open reading frame of *Dictyostelium* 1I encodes a protein which has a predicted cleavable N-terminal signal sequence for targeting into the ER and a C-terminal signal sequence for directing addition of a GPI anchor. Analysis of the sequence upstream from the C-terminal hydrophobic region using the algorithm of Udenfriend and Kodukula [8] and the big-Π predictor [13] indicated that the most probable site for cleavage of the polypeptide chain and attachment of the GPI anchor, the ω residue, was Asn133, which places Ser134 at the ω+1 position and Ala135 at the ω+2 position. This is consistent with analysis of known GPI-anchored proteins and site-directed mutagenesis of others. Asn has been experimentally determined as the ω residue in several GPI-anchored proteins, including *Leishmania major* promastigote surface protease [33], yeast Gas1 [12] and human CD59 [34]. Mutagenesis of the ω residue in alkaline phosphatase [35] and decay accelerating factor [11] has shown that Asn can act as the cleavage/attachment site residue. In the slime mold proteins PsA and gp64 the ω, ω+1 and ω+2 residues have been experimentally determined as Gly-Ser-Ala [3] and Ser-Ser-Ala [4], while in csA they are predicted to be Ser-Ser-Ala. Comparison of the C-terminal sequence of the 1I protein with those of PsA, gp64 and csA reveals that they all have a short hydrophilic spacer region of only four residues between the ω site and the start of the hydrophobic region (see Fig. 4).

It is well documented that the intact, membrane-bound forms of both csA and PsA are resistant to cleavage by bacterial PI-PLC [2,28,29], a criterion commonly used to demonstrate the presence of a GPI anchor on a protein. The reason for this apparent lack of cleavage of the GPI anchor on the *Dictyostelium* proteins is at present unclear. Similarly we could obtain no evidence for the cleavage by bacterial PI-PLC of the GPI anchor on the hexaHis-tagged form of the 1I protein expressed in *D. discoideum*. We therefore utilised the fact that the glucosamine-inositol bond in the GPI anchor is uniquely susceptible to cleavage by nitrous acid, which deaminates the glucosamine residue [36], to demonstrate the presence of a GPI anchor on the 1I protein. Nitrous acid treatment has been shown to cause the partial release of a PI-PLC resistant form of the folate receptor from mammalian cell membranes [30]. Nitrous acid treatment of membranes from *D. discoideum* cells expressing the HexaHis-tagged 1I protein caused partial release of the protein, indicating the presence of a GPI anchor.

In order to confirm that the C-terminal sequence of the 1I protein acted as a GPI anchor addition signal, we replaced the C-terminal signal sequence of the mammalian GPI-anchored protein MDP, which we have extensively characterised [15,16,32], with that from 1I, generating construct MDP-GPI23. The length of the hydrophilic spacer region between the ω site and the hydrophobic C-terminal sequence in most mammalian proteins is between

eight and 12 residues [37]. As noted above, the spacer region in the 1I protein and the other GPI-anchored proteins of cellular slime molds is only four residues. To overcome any potential problem that the spacer region in MDP-GPI23 may be too short to allow GPI anchoring in the mammalian cells, we inserted an extra six residues into the spacer in the MDP-GPI29 construct (see Fig. 4).

When expressed in COS cells, both MDP-GPI23 and MDP-GPI29 fusion proteins were found to be GPI anchored, as shown by digestion with bacterial PI-PLC, and targeted to the plasma membrane in an enzymically active form. To our knowledge this is the first time that a *Dictyostelium* GPI signal sequence has been shown to be functional in mammalian cells. From their size and migration on reducing and non-reducing SDS-PAGE, and 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 MDP-GPI23 or MDP-GPI29. Although we had expected that the short hydrophilic spacer present in construct MDP-GPI23 might have perturbed GPI anchor addition, our analysis of steady state dipeptidase activities revealed no significant differences in the expression levels of MDP-GPI23 and MDP-GPI29, suggesting that the difference in length of the spacer region did not have an adverse effect on GPI anchoring of MDP. The amount of PI-PLC releasable protein at the surface of the COS cells was slightly less for MDP-GPI23 and MDP-GPI29 than for wild type MDP. This is clearly not due to the differing length of the hydrophilic spacer, but may be due to the different ω and ω+1 residues in the constructs as compared with wild type MDP. Asn and Ser, the ω and ω+1 residues in the constructs, have been shown experimentally to lower the efficiency of GPI anchoring in mammalian cells as compared with Ser and Ala, the ω and ω +1 residues in wild type MDP [10,35]. Alternatively, or in addition, the length (18 residues in the 1I protein compared with 14 residues in MDP) and sequence of the hydrophobic domain may affect GPI anchoring in COS cells [7]. However, the totality of all the techniques applied support the major conclusion that Asn133 is the GPI anchor addition site in the 1I protein, even in the absence of mass spectrometry after proteolytic disintegration of the protein.

The calculated size of the mature hexaHis-tagged 1I polypeptide, following removal of the N- and C-terminal signal sequences, is 12.8 kDa. Addition of the GPI anchor would be expected to add approx. 2 kDa to the mature protein, depending on the number and nature of the side chain modifications to the core anchor structure [16]. However, on SDS-PAGE the hexaHis-tagged protein migrated as a strongly expressed doublet at 24 and 27 kDa and a third band at 22kDa. The most likely explanation for this increase in size and the appearance of multiple bands is extensive and variable glycosylation of the 1I protein. Sugar chains account for 40.4% of the mass of PsA [38], 41.3% of that of gp138 [5], and 35.7% of the mass of csA, where O-linked oligosaccharides contribute approx. 10–12 kDa to the apparent molecular mass of the protein [39]. Also 56% of the apparent mass of gp64 has been attributed to glycosylation and the non-specific association of lipids [4]. The mature polypeptide of the 1I protein contains four potential N-linked glycosylation sites, and 28 Ser and Thr residues, representing 25% of the amino acid composition, which may be *O*-glycosylated. Both PsA and csA contain tandem repeats of the tetrapeptide sequence Pro-Thr-Xaa-Thr in which both Thr residues are *O*-glycosylated [28,40] and PsA also contains a Xaa-Pro-Xaa-Xaa motif where at least one Xaa corresponds to a glycosylated Thr [41]. The 1I protein contains a Pro-Thr-Phe-Thr sequence at residues 106–109 and a Thr-Pro-Ala-Gly sequence at residues 129–132 which may be sites for glycosylation. However, digestion with either peptide *N*-glycosidase F or *O*-glycanase in conjunction with neuraminidase did not alter the size of the 22, 24 or 27 kDa polypeptides (data not shown). We have found no reports of successful enzymic deglycosylation of other cellular slime mold glycoproteins, suggesting that carbohydrate modifications resistant to such treatment may well be present on these proteins.

The reasons why specific proteins are GPI-anchored are often unclear. GPI anchors are involved in intracellular sorting, in transmembrane signalling, and in the endocytic process of potocytosis. The presence of a GPI anchor also enables a protein to associate with cholesterol and glycosphingolipid-rich membrane domains, to be more laterally mobile in the plane of the bilayer, and to be selectively released from the membrane by endogenous phospholipases

[42–45]. Northern blots of endogenous mRNA had previously shown that 1I is first expressed between 13 and 15 h of development [14]. However, the function of the 1I protein is currently unknown. Both csA and PsA are expressed during the asexual developmental cycle of *D. discoideum*. Transcription of the csA gene is induced in the preaggregation phase and the protein, which mediates an EDTA-stable form of cell adhesion, is sensitive to release by an endogenous anchor degrading enzyme [46]. The gp64 protein of *P. pallidum* has also been shown to mediate EDTA-stable cell adhesion during development [47]. *Dictyostelium* PsA is first detected on the surface of prespore cells at the multicellular slug stage of development and disappears prior to culmination [48]. The glycoprotein gp138 has been implicated in the sexual development of *D. discoideum* and is specifically expressed on the surface of cells when they acquire competence for sexual cell fusion [5]. It is likely that the addition of GPI anchors to these proteins is crucial for their correct function, and the identification of 1I as a GPI-anchored protein may aid in defining its cellular role.

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References

[1] M.J. McConville, M.A.J. Ferguson, The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes, Biochem. J. 294 (1993) 305–324.

[2] H. Sadeghi, A.M. da Silva, C. Klein, Evidence that a glycolipid tail anchors antigen 117 to the plasma membrane of *Dictyostelium discoideum* cells, Proc. Natl. Acad. Sci. USA 85 (1988) 5512–5515.

[3] P.A. Haynes, A.A. Gooley, M.A.J. Ferguson, J.W. Redmond, K.L. Williams, Post-translational modifications of the *Dictyostelium discoideum* glycoprotein PsA. Glycosylphosphatidylinositol membrane anchor and composition of the O-linked oligosaccharides, Eur. J. Biochem. 216 (1993) 729–737.

[4] R. Manabe, T. Saito, T. Kumazaki, T. Sakaitani, N. Nakata, H. Ochiai, Molecular cloning and the COOH-terminal processing of gp64, a putative cell-cell adhesion protein of the cellular slime mold *Polysphondylium pallidum*, J. Biol. Chem. 269 (1994) 528–535.

[5] H. Fang, M. Higa, K. Suzuki, K. Aiba, H. Urushihara, K. Yanagisawa, Molecular cloning and characterization of two genes encoding gp138, a cell surface glycoprotein involved in the sexual cell fusion of *Dictyostelium discoideum*, Dev. Biol. 156 (1993) 201–208.

[6] P.T. Englund, The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors, Annu. Rev. Biochem. 62 (1993) 121–138.

[7] S. Udenfriend, K. Kodukula, How glycosyl-phosphatidylinositol-anchored membrane proteins are made, Annu. Rev. Biochem. 64 (1995) 563–591.

[8] S. Udenfriend, K. Kodukula, Prediction of ω site in nascent precursor of glycosylphosphatidylinositol protein, Methods Enzymol. 250 (1995) 571–582.

[9] L.D. Gerber, K. Kodukula, S. Udenfriend, Phosphatidylinositol glycan (PI-G) anchored membrane proteins. Amino acid requirements adjacent to the site of cleavage and PI-G attachment in the COOH-terminal signal peptide, J. Biol. Chem. 267 (1992) 12168–12173.

[10] K. Kodukula, L.D. Gerber, R. Amthauer, L. Brink, S. Udenfriend, Biosynthesis of glycosylphosphatidylinositol (GPI)-anchored membrane proteins in intact cells: specific amino acid requirements adjacent to the site of cleavage and GPI attachment, J. Cell Biol. 120 (1993) 657–664.

[11] P. Moran, H. Raab, W.J. Kohr, I.W. Caras, Glycophospholipid membrane anchor attachment. Molecular analysis of the cleavage/attachment site, J. Biol. Chem. 266 (1991) 1250–1257.

[12] C. Nuoffer, A. Horvath, H. Riezman, Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of *Saccharomyces cerevisiae* Gas1 protein, J. Biol. Chem. 268 (1993) 10558–10563.

[13] B. Eisenhaber, P. Bork, F. Eisenhaber, Prediction of potential GPI-modification sites in proprotein sequences, J. Mol. Biol. 292 (1999) 741–758.

[14] A.J. Corney, A.J. Richards, T. Phillpots, B.D. Hames, Developmental regulation of cell-type-enriched mRNAs in *Dictyostelium discoideum*, Mol. Microbiol. 4 (1990) 613–623.

[15] E. Rached, N.M. Hooper, P. James, G. Semenza, A.J. Turner, N. Mantei, cDNA cloning and expression in *Xenopus laevis* oocytes of pig renal dipeptidase, a glycosyl-phosphatidylinositol-anchored ectoenzyme, Biochem. J. 271 (1990) 755–760.

[16] I.A. Brewis, M.A.J. Ferguson, A. Mehlert, A.J. Turner, N.M. Hooper, Structures of the glycosyl-phosphatidylinositol anchors of porcine and human membrane dipeptidase. Interspecies comparison of the glycan core structures and further structural studies on the porcine anchor, J. Biol. Chem. 270 (1995) 22946–22956.

[17] Z.B. Ogel, M.J. McPherson, Efficient deletion mutagenesis by PCR, Protein Eng. 5 (1992) 467–468.

[18] S. Keynan, N.M. Hooper, A.J. Turner, Directed mutagenesis of pig renal membrane dipeptidase. His^{219} is critical but the

DHXXH motif is not essential for zinc binding or catalytic activity, FEBS Lett. 349 (1994) 50–54.
[19] I.J. White, A. Souabni, N.M. Hooper, Comparison of the glycosyl-phosphatidylinositol cleavage/attachment site between mammalian cells and parasitic protozoa, J. Cell Sci. 113 (2000) 721–727.
[20] W. Nellen, C. Silan, R. Firtel, DNA-mediated transformation in *Dictyostelium discoideum*: regulated expression of an actin gene fusion, Mol. Cell. Biol. 4 (1984) 2890–2898.
[21] A.E. Early, J.G. Williams, Two vectors which facilitate gene manipulation and a simplified transformation procedure for *Dictyostelium discoideum*, Gene 59 (1987) 99–106.
[22] D.G. Wilkinson, J. Wilson, B.D. Hames, Spore coat protein synthesis during development of *Dictyostelium discoideum* requires a low-molecular-weight inducer and continued multicellularity, Dev. Biol. 107 (1985) 38–46.
[23] N.M. Hooper, M.G. Low, A.J. Turner, Renal dipeptidase is one of the membrane proteins released by phosphatidylinositol-specific phospholipase C, Biochem. J. 244 (1987) 465–469.
[24] N.M. Hooper, A.J. Turner, Isolation of two differentially glycosylated forms of peptidyl-dipeptidase A (angiotensin converting enzyme) from pig brain: a re-evaluation of their role in neuropeptide metabolism, Biochem. J. 241 (1987) 625–633.
[25] G.M. Littlewood, N.M. Hooper, A.J. Turner, Ectoenzymes of the kidney microvillar membrane. Affinity purification, characterization and localization of the phospholipase C-solubilized form of renal dipeptidase, Biochem. J. 257 (1989) 361–367.
[26] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, B.J. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[27] G. von Heijne, A new method for predicting signal sequence cleavage sites, Nucleic Acids Res. 14 (1986) 4683–4690.
[28] A.A. Gooley, R. Marshchalek, K.L. Williams, Size polymorphisms due to changes in the number of O-glycosylated tandem repeats in the *Dictyostelium discoideum* glycoprotein PsA, Genetics 130 (1992) 749–756.
[29] J. Stadler, T.W. Keenan, G. Bauer, G. Gerisch, The contact site A glycoprotein of *Dictyostelium discoideum* carries a phospholipid anchor of a novel type, EMBO J. 8 (1989) 371–377.
[30] X. Wang, G. Jansen, J. Fan, W.J. Kohler, J.F. Ross, J. Schornagel, M. Ratnam, Variant GPI structure in relation to membrane-associated functions of a murine folate receptor, Biochemistry 35 (1996) 16305–16312.
[31] P. Moran, I.W. Caras, Proteins containing an uncleaved signal for glycophosphatidylinositol membrane anchor attachment are retained in a post-ER compartment, J. Cell Biol. 119 (1992) 763–772.
[32] S. Keynan, N.T. Habgood, N.M. Hooper, A.J. Turner, Site-directed mutagenesis of conserved cysteine residues in porcine membrane dipeptidase. Cys 361 alone is involved in disulphide-linked dimerization, Biochemistry 35 (1996) 12511–12517.
[33] P. Schneider, M.A.J. Ferguson, M.J. McConville, A. Mehlert, S.W. Homans, C. Bordier, Structure of the glycosylphosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease, J. Biol. Chem. 265 (1990) 16955–16964.
[34] Y. Sugita, Y. Nakano, E. Oda, K. Noda, T. Tobe, N.-H. Miura, M. Tomita, Determination of carboxyl-terminal residue and disulphide bonds of MACIF (CD59), a glycosylphosphatidylinositol-anchored membrane protein, J. Biochem. 114 (1993) 473–477.
[35] R. Micanovic, L.D. Gerber, J. Berger, K. Kodukula, S. Udenfriend, Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase, Proc. Natl. Acad. Sci. USA 87 (1990) 157–161.
[36] M.A.J. Ferguson, Chemical and enzymatic analysis of glycosyl-phosphatidylinositol anchors, in: N.M. Hooper, A.J. Turner (Eds.), Lipid Modification of Proteins: a Practical Approach, IRL Press, Oxford, 1992, pp. 191–230.
[37] Y. Furukawa, K. Tsukamoto, H. Ikezawa, Mutational analysis of the C-terminal signal peptide of bovine liver 5′-nucleotidase for GPI anchoring: a study on the significance of the hydrophilic spacer region, Biochim. Biophys. Acta 1328 (1997) 185–196.
[38] A.E. Early, J.G. Williams, H.E. Meyer, S.B. Por, E. Smith, K.L. Williams, A.A. Gooley, Structural characterization of *Dictyostelium discoideum* prespore-specific gene d19 and of its product, cell surface glycoprotein PsA, Mol. Cell. Biol. 8 (1988) 3458–3466.
[39] A. Noegel, G. Gerisch, J. Stadler, M. Westphal, Complete sequence and transcript regulation of a cell adhesion protein from aggregating *Dictyostelium* cells, EMBO J. 5 (1986) 1473–1476.
[40] N.E. Zachara, N.H. Packer, M.D. Temple, M.B. Slade, D.R. Jardine, P. Karuso, C.J. Moss, B.C. Mabbutt, P.M. Curmi, K.L. Williams, A.A. Gooley, Recombinant prespore-specific antigen from *Dictyostelium discoideum* is a beta-sheet glycoprotein with a spacer peptide modified by O-linked N-acetylglucosamine, Eur. J. Biochem. 238 (1996) 511–518.
[41] A.A. Gooley, B.J. Classon, R. Marschalek, K.L. Williams, Glycosylation sites identified by detection of glycosylated amino acids released from Edman degradation – the identification of Xaa-Pro-Xaa-Xaa as a motif for Thr-O-glycosylation, Biochem. Biophys. Res. Commun. 178 (1991) 1194–1201.
[42] E. Rodriguez-Boulan, S.K. Powell, Polarity of epithelial and neuronal cells, Annu. Rev. Cell Biol. 8 (1992) 395–427.
[43] D. Brown, The tyrosine kinase connection: how GPI-anchored proteins activate T cells, Curr. Opin. Immunol. 5 (1993) 349–354.
[44] R.G.W. Anderson, The caveolae membrane system, Annu. Rev. Biochem. 67 (1998) 199–225.

[45] N.M. Hooper, Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae, Mol. Membr. Biol. 16 (1999) 145–156.
[46] A.M. da Silva, C. Klein, Cell adhesion in transformed *D. discoideum* cells: expression of gp80 and its biochemical characterization, Dev. Biol. 140 (1990) 139–148.
[47] S. Funamoto, H. Ochiai, Antisense RNA inactivation of gene expression of a cell-cell adhesion protein (gp64) in the cellular slime mold *Polysphondylium pallidum*, J. Cell Sci. 109 (1996) 1009–1016.
[48] L.H. Browne, H. Sadeghi, D. Blumberg, K.L. Williams, C. Klein, Re-expression of 117 antigen, a cell surface glycoprotein of aggregating cells, during terminal differentiation of *Dictyostelium discoideum* prespore cells, Development 105 (1989) 657–664.
[49] A. Barth, A. Muller-Taubenberger, P. Taranto, G. Gerisch, Replacement of the phospholipid-anchor in the contact site A glycoprotein of *D. discoideum* by a transmembrane region does not impede cell adhesion but reduces residence time on the cell surface, J. Cell Biol. 124 (1994) 205–215.