Structure of the CAMPATH-1 antigen, a glycosylphosphatidylinositol-anchored glycoprotein which is an exceptionally good target for complement lysis

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CAMPATH-1 antibodies recognize a unique molecule on human lymphocytes and are unusually efficient at causing cell lysis with homologous complement. They have been successfully used for lymphocyte depletion *in vivo* in a variety of diseases. We find that the antigen is a very small glycosylphosphatidylinositol (GPI)-anchored glycoprotein with a mature peptide comprising only 12 amino acids. It can be separated into two distinct antigenic fractions which differ in their susceptibility to phosphatidylinositol-specific phospholipase C. There is one N-linked glycosylation site, but no evidence for O-glycosylation despite the presence of several serine and threonine residues. The antibodies were found to bind, albeit with a generally reduced affinity, to a proteolytic fragment containing the C-terminal tripeptide and the GPI anchor. We postulate that one of the reasons why the CAMPATH-1 antibodies are so good for cell lysis is because they bind to an epitope which is likely to be very close to the lipid bilayer.

INTRODUCTION

The CAMPATH-1 antigen (CDw52) is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein which is abundantly expressed on virtually all human lymphocytes (Hale et al., 1983, 1990). Monoclonal antibodies against this antigen are remarkably effective for complement-mediated lysis (Bindon et al., 1988a). CAMPATH-1M (IgM) has been widely used for treatment *in vitro* of allogeneic bone marrow to deplete the donor T cells which cause graft-versus-host disease, normally a frequent and serious complication of marrow transplantation (Hale et al., 1988a). CAMPATH-1G (rat IgG2b) and the reshaped version CAMPATH-1H (human IgG1) are also able to promote cell-mediated killing (antibody-dependent cellular cytotoxicity; ADCC) (Hale et al., 1987; Riechmann et al., 1988). They proved to be very effective for lymphocyte depletion *in vivo* and are being tested for treatment of lymphoma, transplant rejection and various autoimmune diseases (Hale et al., 1988b; Dyer et al., 1989; Mathieson et al., 1990; Friend et al., 1991). The unusual susceptibility of the CAMPATH-1 antigen as a target cannot simply be explained by its abundance (approx. 5×10^5 molecules/lymphocyte), since even small (sub-saturating) amounts of antibody are lytic (Bindon et al., 1988a). Searching for an explanation, we have analysed the primary structure of this antigen and discovered that it is unusual among glycoproteins in being remarkably small (Xia et al., 1991). The antigen had glycolipid-like properties, being extractable with chloroform/methanol. Treatment of cells with phosphatidylinositol-specific phospholipase C (PI-PLC) reduced their level of antigen expression, suggesting that the antigen is GPI-anchored. The purified antigen was heterogeneous with an apparent molecular mass of 21–28 kDa. Its antigenic epitope was heat stable but sensitive to mild alkali. Treatment with *N*-Glycanase or Pronase reduced it to less than 6 kDa, but it could still be recognized by CAMPATH-1 antibodies. N-terminal protein sequencing revealed a short peptide of 11 or 12 residues (GQNDTSQTSSPS), with one N-glycosylation site at Asn-3. The protein sequence deduced by cDNA cloning contained 37 amino acids plus a 24-residue leader sequence. The N-terminal portion agrees perfectly with the actual protein sequence and the C-terminal portion was typical of a hydrophobic GPI-attachment signal sequence which is excised when a GPI anchor is attached.

The attachment site was predicted to be Ser-12 by comparison with other GPI-anchored proteins (Ferguson and Williams, 1988; Cross, 1990). The nature of the antigenic epitope (whether carbohydrate or protein) was not clear. Several other lymphocyte antigens are now known to be GPI-anchored. Indeed the GPI anchor is a common structure throughout phylogeny and many different variants have been discovered (Ferguson and Williams, 1988; Ferguson, 1991, 1992a). GPI membrane anchors have been implicated in protein targeting and signal-transduction events. In the case of CAMPATH-1, the GPI anchor constitutes a significant proportion of the entire glycoprotein structure. Analysis of the CAMPATH-1 antigen might help us to understand more about this intriguing class of molecules, as well as to explain why it is such a good target for therapy.

EXPERIMENTAL

Antigen purification and fragmentation

CAMPATH-1 antigen was purified from human spleens by a modified chloroform/methanol extraction followed by affinity chromatography (Xia et al., 1991). Deoxycholate used to solubilize the antigen during column chromatography was removed by ultrafiltration using a PM10 membrane (Amicon). Alternatively, 1% (w/v) Zwittergent 10 was equally effective but easier to

Abbreviations used: AChE, acetylcholinesterase; GPI, glycosylphosphatidylinositol; MAG; mono-alkylglycerol; PI-PLC, phosphatidylinositol-specific phospholipase C; PVDF, poly(vinylidene difluoride); TMS, trimethylsilyl; Dns, dansyl.

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remove by dialysis due to its higher critical micelle concentration. Antigen which eluted from the affinity column at a pH value of 11.5 was neutralized, ultrafiltered or dialysed against water, then freeze-dried and resuspended in a small volume of water and stored at −30 °C. If required, residual lipid and protein contaminants were removed as follows. Affinity-purified antigen (in water) was thoroughly mixed with an equal volume of water-saturated butanol for 5 min. The interface and aqueous layer were pooled and fractionated by chromatography on octyl-Sepharose. The sample was adjusted to contain 100 mM ammonium acetate, pH 5.5. It was filtered through a nylon membrane and applied to a column (1 cm × 5 cm) of octyl-Sepharose 4B at a flow rate of 5 ml/h. The column was washed with buffer A [100 mM ammonium acetate, pH 5.5, containing 5% (v/v) propan-1-ol] and eluted with a gradient from buffer A to buffer B [60% (v/v) propan-1-ol in water]. Selected fractions were analysed for antigen titre, inositol content and presence of carbohydrate, and the concentration of propan-1-ol was measured by refractive index. Peak fractions were pooled for further analysis.

Pronase-digested antigen was isolated in the same way. The pooled interface and aqueous phase from the butanol extract was dried and resuspended in 0.1 M NH_4HCO_3, pH 8. Pronase (10 mg/ml in 25 mM $CaCl_2$) was added to achieve a final concentration of 1 mg/ml and the mixture was incubated at 37 °C for 16 h. The mixture was acidified to pH 5 with 2 M acetic acid and propan-1-ol was added to a final concentration of 5% before fractionation by chromatography on octyl-Sepharose as described above.

Solid-phase enzyme-linked assay

Antigen samples underwent doubling dilutions in methanol in microtitre plates and were left at 37 °C to dry. The plates were blocked with PBS containing 20% (v/v) foetal-calf serum and 0.02% sodium azide. Antigen was detected using biotinylated CAMPATH-1M and streptavidin–peroxidase complex (Amersham) or unlabelled CAMPATH-1 antibodies followed by peroxidase-linked anti-(rat Ig) antibodies (Xia et al., 1991). PBS containing 0.5% Tween 20 was used as wash buffer in all the assays described here, though in other experiments PBS containing 0.1% BSA could be used with equivalent results. Subsequently, a more sensitive assay was developed as follows. Dilutions (2-fold) of antigen in 50% (v/v) methanol were allowed to dry overnight at room temperature in poly(vinylidene chloride) flexible flat-bottomed microtitre plates (Titertek 'high-activated'). The plates were blocked with PBS containing 2% (w/v) BSA and 0.02% sodium azide. Antigen was detected with unlabelled CAMPATH-1H and alkaline phosphatase-conjugated goat anti-(human IgG) antibody (Sigma). Colour was developed using an amplified substrate system (Self, 1985).

An inhibition assay was used to detect and quantify soluble antigen. Samples of purified antigen or soluble peptides were serially diluted in CAMPATH-1 antibody at a sub-saturating concentration (1–2 μg/ml in PBS) and incubated at 20 °C for 2 h. (No detergent was added, so the 'soluble' antigen was likely to be in the form of micelles.) The mixture was transferred to a plate already coated with spleen extract and the binding of free antibody was measured using peroxidase-conjugated rabbit anti-(rat Ig) antibody.

SDS/PAGE and Western blotting

Slab SDS/PAGE was carried out according to the method of Laemmli (1970). After electrophoresis, the gel was blotted on to a poly(vinylidene difluoride) (PVDF) membrane. The membrane was immersed in 0.1% Coomassie Blue in 50% (v/v) methanol for 5 min, and destained in 45% (v/v) methanol/7% (v/v) acetic acid. The staining pattern was recorded, then the membrane was totally destained in 100% methanol and immunostained with CAMPATH-1G and peroxidase-conjugated anti-(rat Ig) antibody (Xia et al., 1991).

T.l.c.

T.l.c. was carried out using silica-gel 60 sheets (Camlab); first in a tank equilibrated with chloroform/methanol/0.2% KCl (5:5:1.5, by vol.), and then in a tank equilibrated with butan-1-ol/pyridine/0.2% KCl (9:6:4, by vol.) (McConville and Bacic, 1989). The sheet was dried and immunostained as for a Western blot.

Orcinol staining for carbohydrate detection

A silica-gel sheet was loaded with samples and sprayed with 0.2% orcinol in H_2SO_4/H_2O (3:1, v/v) until the plate was damp. The plate was then heated at 100 °C for 10–15 min.

Digestion with proline endopeptidase

Samples of native or Pronase-treated antigen were incubated with proline endopeptidase from *Flavobacterium meningosepticum* (ICN, 50 units/ml in PBS/azide) at 37 °C for 1–4 days. Antigen activity was assayed by e.l.i.s.a. using biotin-labelled CAMPATH-1M and streptavidin–peroxidase as described above.

Digestion using PI-PLC

Samples (1 ml) were incubated at 37 °C for 16 h in 20 mM Tris acetate, pH 7.4, containing 0.1% Triton X-100 in the presence or absence of PI-PLC from *Bacillus thuringiensis*. They were extracted three times with 2 ml of toluene, adjusted to contain 100 mM ammonium acetate and 5% (v/v) propan-1-ol (buffer A), and then mixed with 0.5 ml of octyl-Sepharose gel. After 30 min at 20 °C the mixture was transferred to a small chromatography column and eluted with 1 ml of buffer A. The unbound material was pooled and freeze-dried and the column was further eluted with 2.25 ml of 50% (v/v) propan-1-ol. These fractions ('bound') were also pooled and freeze-dried and samples were assayed by e.l.i.s.a. for antigenic activity.

Triton X-114 phase separation

Precondensed Triton X-114 (12%, w/v) in TBS (10 mM Tris/HCl, 150 mM NaCl, pH 7.4) was added to the samples to give a final concentration of 2% (Bordier, 1981). The samples were left on ice for 10 min with occasional vortexing and then incubated at 37 °C for 10 min. After brief centrifugation, the aqueous (upper) phase was collected and analysed by e.l.i.s.a. and Western blotting.

Carbohydrate and lipid analysis

Analyses were performed with a Hewlett–Packard 5890-5970 g.c.–m.s. system. The derivatized components were separated by g.c. using an SE-54 column (0.24 mm × 30 m, Alltech). Inositol was measured by selected ion monitoring (Smith et al., 1987) following acid hydrolysis (6 M HCl, 110 °C for 16 h) and trimethylsilyl (TMS) derivatization. Monosaccharides were

measured following methanolysis and TMS derivatization. Fatty acid and alkylglycerol content were measured simultaneously, although only qualitatively, by the same procedure. Details of the analysis were exactly as described before (Ferguson, 1992b).

Protein analysis

Samples for amino acid analysis were hydrolysed in 6 M HCl vapour for 16–24 h at 110 °C under vacuum and analysed using either a PicoTag or Alpha Plus (Pharmacia) system. Peptides were sequenced on either an Applied BioSystem model 477A pulsed-liquid sequencer or a model 470A gas-phase sequencer attached to a 120A phenylthiohydantoin amino acid analyser. Dansylation, hydrolysis and t.l.c. were carried out as described by Perham (1978).

Pronase digestion of CD59 antigen

Affinity-purified CD59 antigen from human erythrocyte membranes (Davies et al., 1989) was kindly supplied by Dr. M. Wing (Molecular Immunopathology Unit, Cambridge, U.K.). The CD59 antigen (approx. 30 μg/ml in PBS) was mixed with an equal volume of Pronase (1 mg/ml in PBS) or PBS alone (control) and incubated at 37 °C for 20 min. Samples were titred in methanol on microtitre plates and assayed for antibody binding using CAMPATH-1, CD59 or an irrelevant isotype-matched control antibody followed by peroxidase-conjugated rabbit anti-(rat Ig) antibody as described above.

RESULTS

Analysis of the native antigen

Two batches of affinity-purified antigen were assayed for amino acids, ethanolamine, inositol and carbohydrates. We found significant amounts of the amino acid residues expected from the known sequence (G,Q,N,D,T,S,P) plus the typical components of a GPI anchor (ethanolamine, mannose, *myo*-inositol, fatty acids) and of an N-linked oligosaccharide (GlcNAc, Man, Fuc, Gal, sialic acid). However, the molar ratios were not integral and some unexpected amino acids were also present (at lower yield), indicating that the antigen was probably not completely pure. Since it is so small, even a trace of contamination (in molar terms) with another protein (e.g. antibody leached from the affinity column) would be noticeable. Further purification by butanol extraction and chromatography on an octyl-Sepharose column was designed to remove lipid and protein contaminants. On a conventional column (1 cm × 5 cm), a single peak of antigenic activity was eluted at 28–35% propan-1-ol. However, on a longer column (0.5 cm × 20 cm), this was just resolved into two peaks which were analysed separately (Figure 1).

Sequence analysis of both peaks gave GQ_DTSQTSSP(S), the same result as obtained before for the affinity-purified antigen (Xia et al., 1991). The blank at cycle 3 is due to N-linked carbohydrate. As before, there was a drop in yield at each serine residue (not unusual) and for this reason, identification of Ser-12 in peak I was equivocal.

The two antigenic fractions were analysed for sensitivity to PI-PLC (Table 1). Peak I was sensitive, whereas peak II was resistant to digestion. This result could be explained if peak II has an additional fatty acid residue attached to the inositol ring, since this substitution is known to inhibit the action of PI-PLC (Roberts et al., 1988b; Walter et al., 1990). Semi-quantitative lipid analysis (results not shown) indicated that peak II contained significantly more palmitate ($C_{16:0}$) than peak I and this would be consistent with its retention on the octyl-Sepharose column. It seems likely that the antigen in peak I does not bind efficiently to microtitre plates after PI-PLC treatment to remove the lipid moiety, but we cannot rule out the possibility that a critical epitope is destroyed. Several other attempts to detect solubilized antigen using Triton X-114 extraction and by Western blotting also gave negative results, even when the blot was 'fixed' with glutaraldehyde. A similar phenomenon has been reported for the related mouse lymphocyte antigen J11d (Alterman et al., 1990).

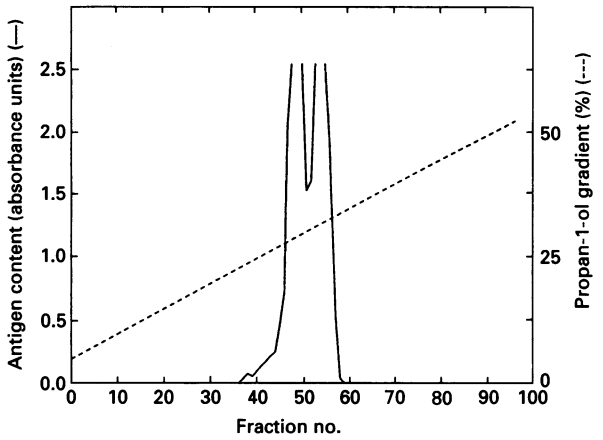

Figure 1 Chromatography of CAMPATH-1 antigen on an octyl-Sepharose column

Affinity-purified antigen was extracted with butanol and applied to a column (0.5 cm × 20 cm) of octyl-Sepharose and eluted with a gradient of propan-1-ol as described in the text. Fractions were assayed for antigenic activity by e.l.i.s.a. Fractions 47–50 (peak I) and 52–56 (peak II) were collected for further analysis.

Table 1 Sensitivity of native CAMPATH-1 antigen to PI-PLC

Samples of native antigen separated by chromatography on an octyl-Sepharose column were treated with PI-PLC at 37 °C for 24 h. They were then fractionated by batchwise binding to octyl-Sepharose and the antigenic activity in the bound and unbound fractions was measured by e.l.i.s.a. using CAMPATH-1H.

	Absorbance	
	Peak I	Peak II
Untreated control		
Bound	1.23	1.62
Unbound	0.00	0.00
PI-PLC treated		
Bound	0.00	2.05
Unbound	0.00	0.00

Analysis of Pronase-treated antigen

Previous experiments using CAMPATH-1M had suggested that antigenic activity was unaffected by proteolytic digestion, although the molecule was cleaved by broad-acting proteases (e.g. Pronase, ficin, bromelain) (Hale et al., 1990). We therefore purified and analysed the digestion product after Pronase treatment in order to examine the antigenic epitope in more detail.

Affinity-purified antigen was extracted with butanol to remove residual detergent and contaminating lipids, then digested over-

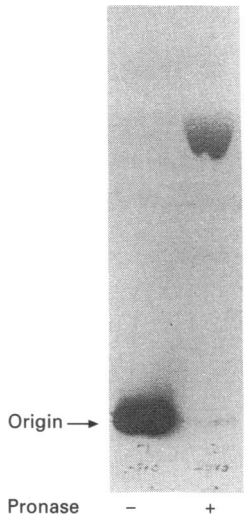

Figure 2 T.l.c. of native and Pronase-treated CAMPATH-1 antigen

Affinity-purified antigen was treated with Pronase (+) or buffer alone (−). Samples were chromatographed on a silica plate as described in the text and immunostained with CAMPATH-1M.

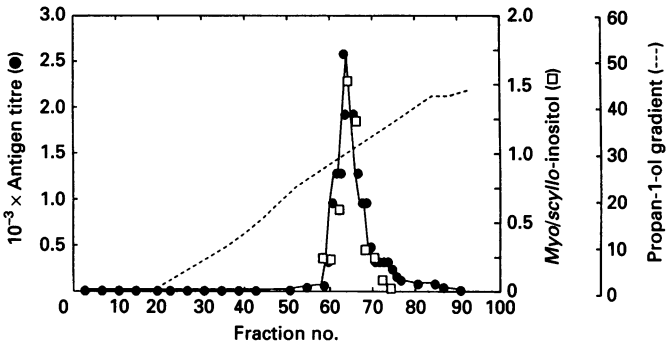

Figure 3 Chromatography of Pronase-treated CAMPATH-1 antigen on an octyl-Sepharose column

Affinity-purified antigen was extracted with butanol and treated with Pronase. The digest was applied to a column (1 cm × 5 cm) of octyl-Sepharose and eluted with a gradient of propan-1-ol. Fractions were assayed for antigenic activity by e.l.i.s.a. and for *myo*-inositol and carbohydrate content. Fractions 60–70 were pooled for further analysis.

night with Pronase. Gel electrophoresis and Western-blot analysis confirmed that digestion was complete. When either the native or the Pronase-treated antigen were extracted with Triton X-114, no antigenic activity could be detected in the aqueous phase. T.l.c. demonstrated that the Pronase-treated antigen behaved like a glycolipid, having a mobility of approx. 0.74 relative to the solvent front, whereas the native antigen remained at the origin (Figure 2). These results suggested that the Pronase-treated antigen retained the GPI anchor and accordingly it was purified by chromatography on an octyl-Sepharose column (Figure 3). The antigen eluted with 28–35% propan-1-ol and this coincided with a peak containing *myo*-inositol and carbohydrate (detected by orcinol staining). Unlike the native antigen (Figure 1), the Pronase-treated antigen did not resolve into two active

Table 2 Analysis of the CAMPATH-1 antigen after digestion with Pronase and chromatography on an octyl-Sepharose column

Unbound and bound fractions collected from the octyl-Sepharose column were pooled, dried down and redissolved in 400 μl of water. Aliquots (20 μl) (5% of total) were taken from each pool and mixed with 2 nmol of *scyllo*-inositol and 2 nmol of norleucine as internal standards. 40% of the mixture was used for amino acid and carbohydrate/lipid analysis respectively, and 10% for inositol analysis. Abbreviations: EtN, ethanolamine; ND, not done; +, clearly positive (equivalent to 10–100 nmol). Values given in parentheses indicate molar ratios relative to *myo*-inositol.

	N-linked oligosaccharide etc. from fractions 4–12 (nmol)	Antigenic fragment from fractions 59–68 (nmol)
Antigen titre	< 1:40	> 1:5120
Amino-acid analysis		
Ser	ND	94 (1.9)
Pro	ND	40 (0.8)
Others	ND	< 11 (< 0.2)
EtN	ND	104 (2.1)
myo-Inositol	15	49 (1.0)
Monosaccharide and lipid (by methanolysis/TMS)		
Man	226	56 (1.1)
Fuc	118	0
Gal	342	0
Glc	308	0
GlcNAc	410	0
GalNAc	0	0
NeuAc	382	0
$C_{16:0}$	ND	+
$C_{17:0}$	ND	Trace
$C_{18:0}$	ND	+
$C_{18:1}$	ND	+
$C_{20:0}$	ND	Trace
$C_{18:0}$-MAG	ND	Trace
$C_{18:1}$-MAG	ND	Trace

peaks, probably because the shorter column was used. Another carbohydrate peak, probably derived from the N-linked oligosaccharide, was found in the unbound fractions. Fractions containing antigen were pooled and used for compositional and sequence analysis (Table 2). The unbound fractions were also analysed for carbohydrate content, but a protein composition would have been uninformative due to the presence of a substantial amount of Pronase.

Analysis by e.l.i.s.a. with a panel of CDw52 antibodies at high concentration (approx. 50 μg/ml) showed that the Pronase-treated antigen could still be detected by all of them, but with varying efficiency (Xia et al., 1993). However, when the antibodies were used at a lower concentration (approx. 2 μg/ml), the binding of CAMPATH-1G and CAMPATH-1H was very weak indeed (approx. 3%). Titration of the antibodies at a fixed antigen concentration showed that the affinity of CAMPATH-1G and CAMPATH-1H for Pronase-treated antigen was substantially reduced compared with the native antigen. On t.l.c. of Pronase-treated antigen, CAMPATH-1G stained the same mobile band as CAMPATH-1M (Figure 2) but much more weakly. This showed that it does bind to the proteolytic fragment and not just to an undigested fraction of the native antigen.

Amino acid analysis of the Pronase-treated antigen showed only serine, proline and ethanolamine in the ratio 2:1:2. Sequence analysis gave the unambiguous result Ser-Pro-Ser-end indicating that the GPI anchor is attached to Ser-12 as predicted. However, the yield of the second serine residue differed according

to the sequencer used. With the gas-phase sequencer we obtained Ser(106 pmol)-Pro(83 pmol)-Ser(85 pmol) but with the pulsed-liquid sequencer we obtained Ser(55 pmol)-Pro(68 pmol)-Ser(19 pmol). It is possible that the C-terminal serine residue, attached to a GPI anchor, may have been 'washed out' during the last cycle in the liquid-phase sequencer. Alternatively, there might be a substituent on the hydroxyl group which is more labile in the gas-phase sequencer.

Treatment of the Pronase antigen with dansyl (Dns) chloride, followed by acid hydrolysis and t.l.c., gave, in addition to a weak spot of Dns-serine, a distinct spot with the same mobilities as Dns-ethanolamine prepared simultaneously (results not shown). The ethanolamine phosphate which presumably links the C-terminal serine to the glycan would be inaccessible to dansylation, so this result suggests that there is indeed a second ethanolamine with a free amino group. By analogy with the GPI anchor of acetylcholinesterase (AchE) (Roberts et al., 1988a), it could be attached to the third mannose of the GPI-core carbohydrate.

Carbohydrate and lipid analysis of the Pronase-treated antigen revealed the typical components of a mammalian GPI anchor, which are mannose, *myo*-inositol and fatty acids. The ratio of proline/mannose/inositol was approx. 1:1:1. This is consistent with an anchor bearing a second ethanolamine phosphate since, although there are three mannose residues in the core, two would then be phosphorylated and so undetectable under our conditions of methanolysis (Ferguson, 1992b). The glucosamine residue would also not be detected due to the relative acid stability of the glucosamine–inositol and inositol–phosphate bonds. The absence of other sugars was notable, and in particular, no *N*-acetylgalactosamine or *N*-acetylglucosamine were detected. This makes it unlikely that there are any O-linked oligosaccharides in the antigen, which is contrary with previous speculations (Hale et al., 1990). Some GPI anchors (e.g. Thy-1) contain additional monosaccharides such as mannose and *N*-acetylgalactosamine (Homans et al., 1988), but these are unlikely to exist in the CAMPATH-1 antigen.

The unbound fractions contained *N*-acetylglucosamine, fucose, galactose, mannose, acetylneuraminic acid and glucose (probably a contaminant) in proportions typical of a complex N-linked oligosaccharide.

Lipid analysis was only semi-quantitative, but a variety of experiments, together with the results of analysis of the native antigen, allow us to exclude certain possibilities and lead to the conclusion that in this respect the CAMPATH-1 antigen is probably similar to other mammalian GPI anchors. After methanolysis and TMS derivatization, several fatty-acid methyl esters ($C_{16:0}$, $C_{18:0}$, $C_{18:1}$) were detected in reasonable yield. However, mono-alkylglycerols (MAGs) ($C_{18:0}$ MAG and $C_{18:1}$ MAG) were detected in only trace amounts. Ceramidephosphatidylinositol anchors have been found in lower organisms; however, in an analysis for their characteristic long-chain bases and amide-linked fatty acids (Ferguson, 1992b), none were found. These results suggest that the GPI anchor comprises diacyl- and/or acyl-(*lyso*)phosphoglycerides.

Reactivity of CAMPATH-1 antibodies with other GPI anchors or synthetic peptides

The above results suggest that the Pronase-treated antigen is a GPI anchor with only a tripeptide (Ser-Pro-Ser) attached. This raised the possibility that the CAMPATH-1 antibodies may be GPI-anchor specific. To test this hypothesis, CAMPATH-1G was used to stain various GPI-anchored molecules [including soluble-form and membrane-form variable surface glycoprotein 118, membrane-form Thy-1 from rat brain and thymus, and membrane-form human erythrocyte AChE] on a PVDF blot after being separated by SDS/PAGE. Although the protein bands of these molecules were clearly visible by staining with Coomassie Blue, none of them could be stained by CAMPATH-1G (results not shown). Affinity-purified CAMPATH-1 antigen and spleen extract were included as positive controls and both could be clearly stained. In another experiment, purified membrane-form CD59 antigen (a GPI-anchored glycoprotein found on human lymphocytes) (Davies et al., 1989) was tested by e.l.i.s.a. Although readily detected by a CD59 antibody, it was not recognized by CAMPATH-1 antibodies. After treatment with Pronase the binding titre with a CD59 antibody was reduced to approx. 2% of the control, but still no binding of CAMPATH-1M or CAMPATH-1G could be detected. These results indicate that the CAMPATH-1 antibodies do not bind to the GPI anchor alone as indeed was expected owing to their specificity for human lymphocytes.

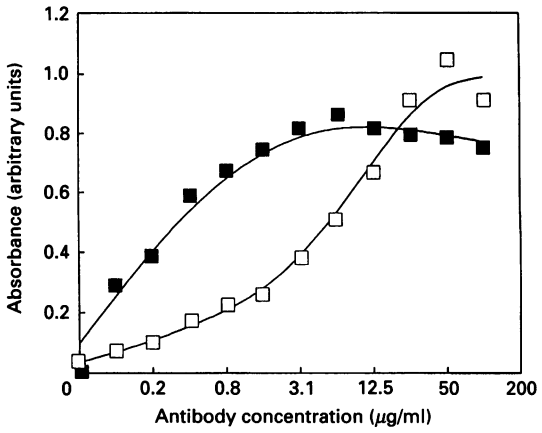

Figure 4 Binding of CAMPATH-1M to synthetic peptide

Microtitre plates were coated with spleen extract containing the native antigen (■) or the synthetic peptide GQNDTSQTSSPS (0.5 mg/ml in PBS) (□). Binding of CAMPATH-1M (up to 100 μg/ml) was measured by e.l.i.s.a. No binding was detected to plates coated with Ser-Pro-Ser or an irrelevant peptide or PBS alone.

To test whether the attached peptide could be antigenic, both the tripeptide (SPS) and the 12-residue peptide corresponding to the complete sequence (GQNDTSQTSSPS) were synthesized. They were used to coat e.l.i.s.a. plates, being diluted in either methanol or 0.1 M sodium carbonate buffer, pH 9.6, or PBS. Neither peptide showed positive binding with CAMPATH-1G and the 12-mer only gave significant binding with CAMPATH-1M when coated at a high concentration (0.5 mg/ml). However, the affinity, as judged by titration of the antibody, was low compared with the native antigen (Figure 4).

Since direct coupling may not have given ideal presentation of an antigenic epitope, the peptides were tested for any inhibition of the binding of CAMPATH-1G (1 μg/ml) or CAMPATH-1M (1 μg/ml) to the native antigen coated on an e.l.i.s.a. plate (Figure 5). Neither of the peptides showed any inhibition even at a concentration much higher than the control samples of native or Pronase-treated antigen. In other experiments the 12-mer peptide was coupled to keyhole limpet haemocyanin using either glutaraldehyde or carbodiimide. The conjugates were diluted to 10 μg/ml in PBS and adsorbed on to microtitre plates. They could readily be recognized by polyclonal antisera (rabbit or rat) raised against them, but not by the CAMPATH-1M or CAMPATH-1G antibodies. These results suggest that the peptide

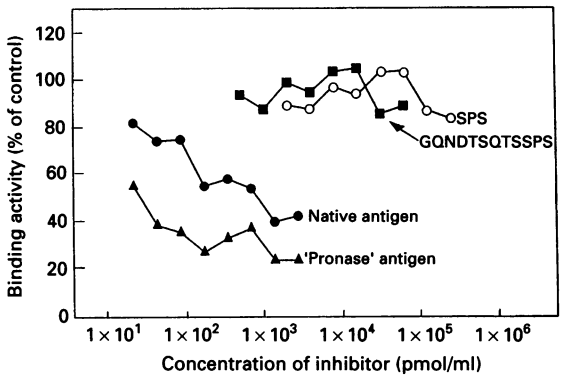

Figure 5 Lack of inhibition by synthetic peptides of CAMPATH-1 binding to antigen

CAMPATH-1M (1 μg/ml) was incubated with samples of peptides, native antigen or Pronase-treated antigen at various concentrations. The mixture was applied to a microtitre plate coated with native antigen. Binding of residual antibody was measured using peroxidase-coupled anti-(rat Ig). The results are expressed as a percentage of the binding with no inhibitor present.

Table 3 Digestion of native and Pronase-treated antigen with proline endopeptidase

Samples were treated with proline endopeptidase or buffer alone at 37 °C for up to 4 days. The antigenic activity was measured by e.l.i.s.a. using biotin-labelled CAMPATH-1M. The titre is the dilution of the reaction mixture which gave half-maximal binding of the labelled antibody.

	Duration of incubation (day)	Titre	
		Native	Pronase-treated
Untreated control	1	440	45
Enzyme treated	1	260	60
Untreated control	4	500	100
Enzyme treated	4	100	7

alone is not sufficient to provide the complete antigenic epitope, although the low-affinity recognition by CAMPATH-1M (Figure 4) implies that it does make a contribution.

Importance of the peptide for the antigenic epitope

Since the CAMPATH-1 antibodies did not appear to react with other GPI-anchored glycoproteins, we surmised that the tripeptide could be an essential, if not sufficient, part of the antigenic epitope. (From the experiments described above, it already seems that the nine N-terminal amino acids also contribute to the binding of CAMPATH-1G.) To test this the native and 'Pronase' antigen were further treated with proline endopeptidase which might cleave the bond between Pro-11 and Ser-12. There was little effect on antigen titre after 24 h digestion, but after 4 days it was largely destroyed (Table 3). Previous work (Yoshimoto et al., 1980) showed that only certain peptides are good substrates for this enzyme; standard proteins (even denatured) were not cleaved and tripeptides were poorly cleaved so it is not remarkable that digestion took so long. However, this experiment supports the notion that the tripeptide Ser-Pro-Ser is a critical part of the antigenic epitope.

DISCUSSION

We have confirmed that the CAMPATH-1 antigen is GPI-anchored and shown that the peptide is attached to the GPI anchor at Ser-12 making it the shortest cell-surface glycoprotein ever found. The antigen can be separated on octyl-Sepharose into two distinct antigenic fractions, which differ in their susceptibility to PI-PLC. Compositional data showing additional $C_{16:0}/C_{18:1}$ in the PI-PLC-resistant fraction is consistent with an acyl-linked fatty acid substitution of the inositol ring, and may explain the incomplete release of the antigen from peripheral blood mononuclear cells treated with PI-PLC (Hale et al., 1990). This intriguing result opens the possibility that the two GPI-anchored forms of the antigen may possess different biological functions.

The Pronase-digested antigen contained only a tripeptide attached to the GPI anchor. This implies that the epitope recognized by the therapeutic CAMPATH-1 antibodies is presumably very close to the cell membrane. As the 12-mer peptide was only very weakly recognized, and other GPI anchors were not recognized at all by the CAMPATH-1 antibodies, the antigenic epitope may comprise the unique combination of these two parts and/or additional unknown substituents. CAMPATH-1G contains eight lysine and arginine residues in its binding site (Riechmann et al., 1988), higher than the average antibody (4.9, calculated from Kabat et al., 1991) without a correspondingly large increase in the number of glutamic or aspartic acid residues. This suggests to us that the epitope may contain a negatively charged residue, possibly one or more of the three phosphate groups likely to be present in the anchor.

Because of the sensitivity of the antigen to mild alkali, it was originally thought that O-linked oligosaccharide(s) may be responsible for the antigenic epitope; there are four serine and two threonine residues in the peptide which could serve as potential glycosylation site(s). Support for this hypothesis was recently presented by Valentin et al. (1992), who found that the antigen is sensitive to treatment with neuraminidase plus endo-*N*-acetylgalactosaminidase ('*O*-Glycanase'). However, no *N*-acetylgalactosamine, typical of O-linked sugar, was found in the Pronase-treated antigen (or in the native antigen). Despite several attempts we have been unable to repeat the observations of Valentin et al. and find that the antigen is resistant to *O*-Glycanase under the conditions they described (M.-Q. Xia, G. Hale and V. Taylor, unpublished work). Other alkali-sensitive structures could be as follows. (a) Hydroxyester-linked fatty acids. Although extremely labile to alkali it seems unlikely that they are antigenic. However, the lipid may be necessary for detection in our binding assays. (b) The second ethanolamine phosphate. It is theoretically plausible that this group is alkali labile due to the intramolecular catalysis possible from the 2′-hydroxyl group of the mannose to which it is attached (analogous to the alkaline cleavage of RNA). (c) O-substitution of serine. Although we found no evidence for O-linked carbohydrate, it is possible that an acetyl, phosphate, or other group may be present. Such a group would have to be labile under the conditions of protein sequencing. Resolution of these possibilities must await detailed structural analysis.

Recent experiments show that the CAMPATH-1 cDNA can be transfected into a variety of mammalian cells including BHK (baby hamster kidney), CHO (chinese hamster ovary) and COS (monkey kidney) cell lines, giving surface expression of CAMPATH-1 antigen (Dr. M. Tone, Department of Pathology

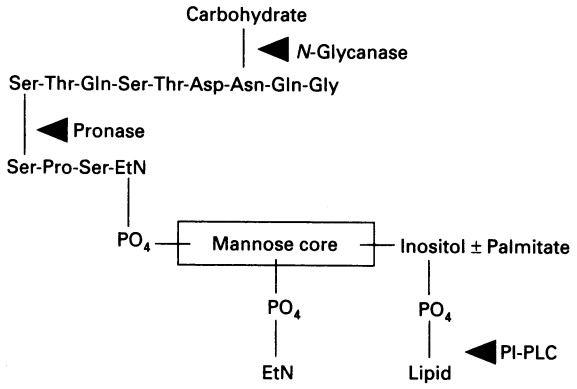

Figure 6 A possible structure for the CAMPATH-1 antigen

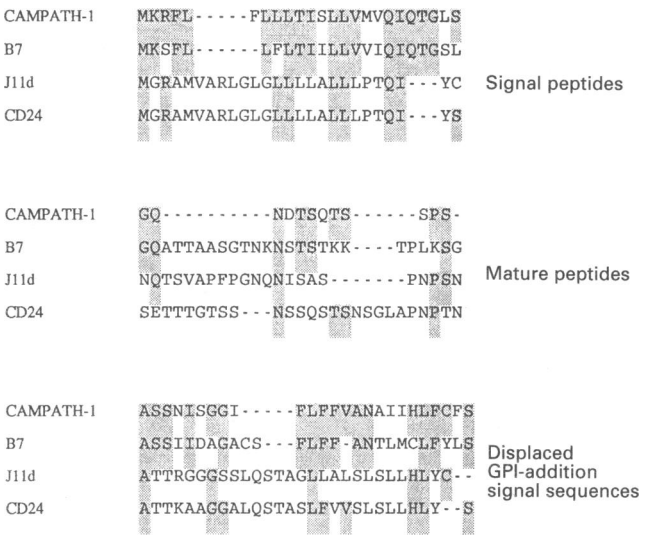

Figure 7 Comparison of the deduced protein sequences of CAMPATH-1 antigen and related molecules

The alignments have been optimized by hand to maximize conserved residues and structural motifs. The site of attachment of the GPI anchor has only been determined for CAMPATH-1; the others are inferred by comparison with known sequences.

Cambridge, U.K. and Dr. J. Tite, Wellcome Research Laboratories, Beckenham, U.K., personal communications). This shows that the antigenic epitope is not a lymphocyte-specific post-translational modification, but suggests that the peptide sequence is critical. A schematic diagram of the antigen, based on known structures and consistent with the data so far known, is shown in Figure 6. There is likely to be substantial heterogeneity in the N-linked oligosaccharide as well as in the fatty acid content.

The cDNAs of another three GPI-anchored molecules with very short peptides have been published: mouse J11d (also called heat-stable antigen) (Kay et al., 1990), human CD24 (Kay et al., 1991; Jackson et al., 1992) and a mouse lymphocyte antigen recognized by an antibody 'B7' (not to be confused with the mouse homologue of the human B7 antigen) (Kubota et al., 1990). The cDNA of CAMPATH-1 antigen is similar in sequence to this 'B7' antigen (59% conservation) but not to CD24 or J11d, which nevertheless are similar to each other (63% conservation). CAMPATH-1 antigen probably has the shortest peptide, though the exact sites of GPI-anchor attachment for the others have not yet been determined. The deduced protein sequences with probable attachment sites are shown in Figure 7. This family of molecules share several biochemical characteristics, such as a glycoplipid-like behaviour in solvent extracts, failure to be detected after iodination (due to lack of tyrosine), and an inability to be stained by Coomassie Blue (because the protein content is so small). Like CAMPATH-1, the antigenic epitope of J11d is heat stable (Springer et al., 1978), but sensitive to mild alkali (Kay et al., 1990), as is the CD24 antigen (Kay et al., 1991).

CD24 antibodies are reported to be cytotoxic with human complement (Slaper-Cortenbach et al., 1987; Humblet et al., 1988; Jackson et al., 1992). The mouse J11d antigen is also a good target for complement lysis (Springer et al., 1978). Lytic effects of the B7 antibody have not been reported. We propose that an antibody-binding site close to the cell membrane may be the crucial factor for these antigens to be such good targets. Their lipid anchor may facilitate bivalent C1q binding (which is essential for C1 activation) by allowing the antigens to be more mobile. However, the short distance between the site of complement activation and the cell membrane may be even more important. The reason for this is that C4 and C3, crucial components of the complement cascade, need to bind to the cell surface via an active thiolester. However, this thiolester has a very short half-life and is soon hydrolysed. Its mean path length is estimated to be about 6 nm (Mardiney et al., 1968). We know that human IgG3, although it activates C1 very efficiently, is less good than IgG1 at causing cell lysis (Bindon et al., 1988b). This isotype has an unusually long hinge (approx. 9 nm), but mutants with conventional hinges give much better lysis (Michaelson et al., 1990). We therefore imagine that C4 and C3 deposition on the cell surface is much more efficient if the activated C1 is generated as close as possible to the cell surface. An alternative hypothesis, that the N-linked oligosaccharide provides a particularly good target for C4 or C3 binding, has been eliminated since we have shown that the Pronase-treated antigen is just as good a target for antibody lysis as the native antigen (Xia et al., 1993).

A search of the literature (Xia, 1992) suggests that many other antigens which are 'good' for complement lysis are glycolipids (Mabry et al., 1985; Saarinen et al., 1985; Welt et al., 1987; Hellstrom et al., 1986, 1990). Their overall structure would be rather similar to this family of small GPI-anchored glycoproteins. However, not all 'good' antigens are glycolipids or glycolipid-like; some are glycoproteins (e.g. Slaper-Cortenbach et al., 1987; Liu et al., 1987; Bindon et al., 1988a). Among them, CD9 and CD20 cross the membrane several times and are thought to have only a very small portion of their peptide exposed extracellularly. Class-I and class-II histocompatibility antigens are among the few examples for typical type-I membrane proteins which are 'good' for complement lysis. However, the recent demonstration that class-I molecules exist as tetramers (Krishna et al., 1992) suggests that a different mechanism could operate, i.e. that of synergistic lysis, since it is known that juxtaposition of two IgG antibodies can give such an improvement in C1q binding as to make even an otherwise poor antigen a good target (Bindon et al., 1985). Clearly there are many different factors which can contribute to the effectiveness of an antigen as a target for complement attack, but the distance of the epitope from the cell membrane may be a particularly significant one.

The CAMPATH-1 antigen is also a very good target for cell lysis *in vivo* and has been successfully used for lymphocyte depletion in a variety of diseases. Studies with different isotypes

showed that complement-mediated lysis alone was not a sufficient explanation for cell clearance, rather cell-mediated killing was implicated. There are rather few other antibodies which give such potent cytotoxicity *in vivo* and most of them seem to recognize glycolipid (ganglioside) antigens, such as GD2 and GD3 (Houghton et al., 1985; Cheung et al., 1987; Irie and Ravindranath, 1990). Although the reasons for them being 'good' might not be the same, this correlation may not be merely coincidental, and more effort should be made to explore the potential of other antibodies against these 'CAMPATH-1 like' targets for serotherapy both *in vitro* and *in vivo*.

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