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Characterization of an antibody to the cross-reacting determinant of the glycosyl-phosphatidylinositol anchor of human membrane dipeptidase

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A polyclonal antiserum raised to the phospholipase C-solubilized form of membrane dipeptidase (EC 3.4.13.11) purified from human kidney was found to cross-react with unrelated trypanosomal and porcine glycosyl-phosphatidylinositol anchored proteins. Those antibodies recognising the cross-reacting determinant (CRD) were isolated by chromatography on a column of immobilized phospholipase C-solubilized porcine aminopeptidase P (EC 3.4.11.9), and the epitopes involved in the recognition were then characterized by immunoelectrophoretic blot analysis and by a competitive ELISA. The phospholipase C-solubilized forms of human and porcine membrane dipeptidase, porcine aminopeptidase P and trypanosome variant surface glycoprotein were recognised by the anti-CRD antiserum, and this recognition was abolished by prior treatment of the proteins with either mild acid or nitrous acid. In contrast, the detergent-solubilized, membrane-forms of human and porcine membrane dipeptidase were not recognised. Of a range of components of the glycosyl-phosphatidylinositol anchor, only inositol 1,2-cyclic monophosphate and the insulin-mimetic disaccharide, glucosaminyl-1,6-inositol 1,2-cyclic monophosphate, inhibited in the micromolar range the binding of the anti-CRD antiserum to immobilized porcine aminopeptidase P. These results indicate that the major epitope recognised by this anti-CRD antiserum is the inositol 1,2-cyclic monophosphate formed on phospholipase C cleavage of the glycosyl-phosphatidylinositol anchor.

Introduction

A variety of eukaryotic proteins have been identified as possessing a glycosyl-phosphatidylinositol (G-PI) membrane anchor (reviewed in Refs. 1 and 2). These proteins, located almost exclusively at the cell surface, are attached to the G-PI anchor via a covalent linkage between the carboxyl group of their C-terminal amino acid and the amino group of phosphoethanolamine in the anchor. This phosphoethanolamine connects via a series of three mannose residues to a non N-acetylated glucosamine residue which in turn links to a phosphatidylinositol molecule. The latter being the sole means of anchorage in the lipid bilayer. This core anchor structure is variably modified in a protein/species-specific manner by the addition of side-chain structures, e.g., galactose residues in the variant surface glycoproteins (VSG) of trypanosomes [3] and *N*-acetylneuraminic acid residues in the scrapie and cellular prion proteins [4].

When incubated in the presence of bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) or when acted on by an endogenous phospholipase C, G-PI-anchored proteins can often be released from the lipid bilayer with concomitant generation of a characteristic structure, the cross-reacting determinant (CRD) [5,6]. Polyclonal antisera raised to the phospholipase C-solubilized forms of a range of G-PI-anchored proteins (VSG of trypanosomes [7], Torpedo acetylcholinesterase [8] and porcine membrane dipeptidase and aminopeptidase P [9]) have been shown to recognise this CRD in other unrelated proteins which possess a G-PI anchor. The major epitope involved in this cross-reactivity has been characterized as inositol 1,2-cyclic monophosphate which is formed by the action of phospholipase C [7–10]. In addition, there appear to be other epitopes which are species-specific and involve

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Abbreviations: AP-P, aminopeptidase P; pAP-P, phospholipase C-solubilized AP-P; CRD, cross-reacting determinant; DP, membrane dipeptidase; mDP, detergent-solubilized membrane-form DP; pDP, phospholipase C-solubilized DP; G-PI, glycosyl-phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein; sVSG, soluble form of VSG.

side-chain modifications to the core anchor structure, e.g., a galactose side chain of variable length in VSG [7]. A polyclonal [11] and a monoclonal antibody [12] have also been generated against the isolated G-PI anchor of VSG. Both these antibodies recognize the soluble and membrane-bound forms of VSG, and thus the site of cross-reactivity cannot involve the inositol 1,2-cyclic monophosphate which is formed on phospholipase C cleavage, but epitopes elsewhere on the anchor structure. Whether either of these antibodies recognise G-PI anchors on unrelated proteins remains to be established.

Available anti-CRD antisera have been used primarily to confirm the presence of a G-PI anchor on a protein following release by either exogenous PI-PLC [for example 13–15] or endogenous phospholipase C (e.g., Refs. 16–18). An anti-CRD antiserum has also been used to demonstrate the cleavage specificity of a phospholipase C isolated from the bacterium *Cytophaga* which cleaves the G-PI anchor of acetylcholinesterase [19]. These antisera may also have potential in studies on the structure and biosynthesis of G-PI anchors [20]. The polyclonal antibody raised to the isolated G-PI anchor of VSG has been shown to block some of the actions of insulin which are mediated by inositol phosphoglycans [11,21]. In addition, polyclonal antisera have been generated against isolated inositol phosphoglycans [22,23], and in one case shown to recognise the G-PI anchor on Thy-1 [23]. Obviously a pre-requisite of using anti-CRD antisera in such studies is a detailed knowledge of the epitopes involved in their cross-reactivity.

In the present study we show that a polyclonal antiserum raised to the PI-PLC-solubilized form of human membrane dipeptidase (DP; dehydropeptidase I, EC 3.4.13.11) [24] cross-reacts with other G-PI anchored proteins. This anti-CRD antiserum has been purified and the epitopes involved in the cross-reactivity determined by immuno-electrophoretic blot analysis and by a competitive ELISA. The results indicate that the major epitope involved in the cross-reactivity with other G-PI anchored proteins by this antiserum is the inositol 1,2-cyclic monophosphate generated on phospholipase C cleavage of the anchor. Other minor epitopes, possibly involving side-chain modifications to the core anchor structure, appear to be protein-specific. In addition, this anti-CRD antiserum recognises the recently synthesized insulin-mimetic disaccharide, glucosaminyl-1,6-inositol 1,2-cyclic monophosphate [25].

Materials and Methods

Materials

Variant surface glycoprotein 117 (sVSG) was a gift from Dr. M.A.J. Ferguson, University of Dundee, Dundee, UK. Glucosaminyl-1,6-inositol 1,2-cyclic monophosphate was a gift from Dr. M. d'Alarcao, Tufts University, MA, USA. All other materials were obtained from sources previously noted. Dipeptidase (DP) was purified from human and porcine kidney cortex by affinity chromatography on cilastatin-Sepharose after solubilization with either bacterial PI-PLC (phospholipase-solubilized dipeptidase; pDP) or n-octyl β-D-glucopyranoside (detergent-solubilized membrane-form dipeptidase; mDP) [14,24,26]. Aminopeptidase P (AP-P; EC 3.4.11.9) was purified from porcine kidney cortex after solubilization with bacterial PI-PLC (pAP-P) as described in Hooper et al. [15]. Protein was determined using bicinchoninic acid in a microtitre plate assay with bovine serum albumin as standard.

Production and purification of antibody

An antiserum was raised against purified human pDP in New Zealand white rabbits. An IgG fraction was prepared from the serum by affinity chromatography on a column of Protein A-Sepharose. The antibodies recognizing the CRD component were purified from the IgG fraction by affinity chromatography on a column of pig kidney pAP-P immobilized on CNBr-activated Sepharose as described in Matsas et al. [27].

Mild acid and nitrous acid treatments

The inositol 1,2-cyclic monophosphate, formed on phospholipase C cleavage, was selectively decyclized by incubation of the protein in 1 M HCl for 30 min at 23°C [7]. After neutralization with NaOH, samples were subjected to immunoelectrophoretic blot analysis. Proteins were deaminated by treatment with 0.25 M sodium acetate, 0.25 M $NaNO_2$ (pH 4.0) for 3 h at 23°C [7]. Control samples were treated with 0.25 M sodium acetate, 0.25 M NaCl (pH 4.0). After neutralization, samples were acetone-precipitated and then subjected to immunoelectrophoretic blot analysis.

SDS polyacrylamide gel electrophoresis and immunoelectrophoretic blot analysis

SDS polyacrylamide gel electrophoresis was performed with a 7–17% polyacrylamide gradient as described previously [28]. Immuno-electrophoretic blot analysis was carried out with Immobilon P (poly(vinylidene difluoride)) membranes and a ^{125}I-labelled second antibody as detailed previously [24,29].

Competitive ELISA

The CRD was characterized by measuring the ability of compounds to inhibit the binding of the antibody to pAP-P immobilized in micro-titre plate wells. Dilutions of the test compound in 0.1% (w/v) BSA in phosphate-buffered saline (20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.25 M NaCl (pH 7.4)) were pre-incubated with antibody for 2 h at 23°C. Samples were centrifuged at $8800 \times g$ for 10 min, then triplicate 50 μl

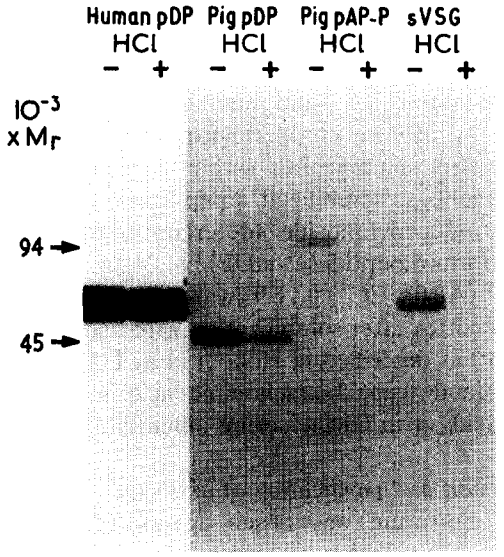

Fig. 1. Immunoelectrophoretic blot of G-PI-anchored proteins with the crude antiserum. Samples of phospholipase C-solubilised proteins (5 μg protein per track) were prepared and analysed as described in Materials and Methods. After electrophoretic transfer to Immobilon P membranes, the tracks were blotted with the crude antiserum. Bound antiserum was detected with a ^{125}I-labelled second antibody, followed by autoradiography.

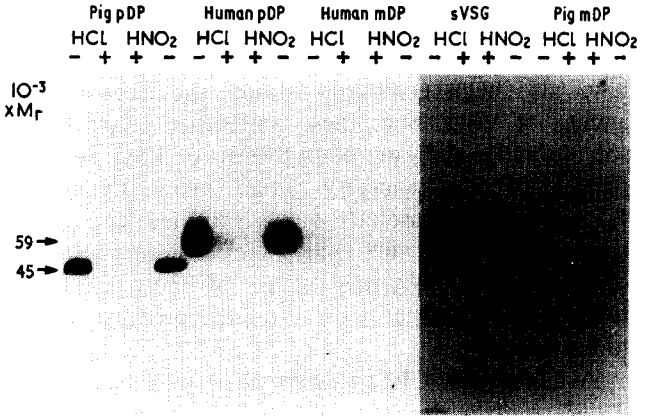

Fig. 2. Immunoelectrophoretic blot of G-PI-anchored proteins with the anti-CRD antiserum. Samples of purified proteins (5 μg protein per track) were prepared and analysed as described in Materials and Methods. After electrophoretic transfer to Immobilon P membranes, the tracks were blotted with the anti-CRD antiserum. Bound antiserum was detected with a ^{125}I-labelled second antibody, followed by autoradiography.

portions were transferred to pre-coated micro-titre plate wells and incubated for 16–20 h at 4°C. The plates were washed, bound antibody detected with biotinylated rabbit immunoglobulin and streptavidin-biotinylated horseradish peroxidase complex and the assay developed with 2, 2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) as detailed in Hooper et al. [9].

Results

Cross-reactivity of the crude antiserum with other G-PI-anchored proteins

A polyclonal antiserum raised to PI-PLC-solubilised, affinity purified human membrane dipeptidase (pDP) was shown by immuno-electrophoretic blot analysis to recognise the purified protein (Fig. 1). Treatment of purified human pDP with 1 M HCl prior to electrophoresis did not affect the recognition by the crude antiserum. The antiserum was also found to cross-react with other G-PI-anchored proteins that had been solubilised with phospholipase C (Fig. 1). In the case of pig pAP-P and trypanosome sVSG this cross-reactivity was selectively abolished by prior treatment of the proteins with 1 M HCl. This treatment however only partially decreased the recognition of pig pDP by the crude antiserum (Fig. 1). In order to characterise further the epitopes involved in the CRD, the population of antibodies recognising the CRD were purified from the crude antiserum by chromatography on a column of immobilized pig pAP-P (see Methods section for details). The population of antibodies that bound to the immobilized pAP-P were eluted with 0.2 M glycine, (pH 2.3), and are referred to as the anti-CRD antiserum.

Characterisation of the epitopes involved in the CRD by immunoelectrophoretic blot analysis

On immunoelectrophoretic blot analysis the anti-CRD antiserum was shown to recognise phospholipase C-solubilised forms of pig DP, human DP, trypanosome VSG (Fig. 2) and pig AP-P (results not

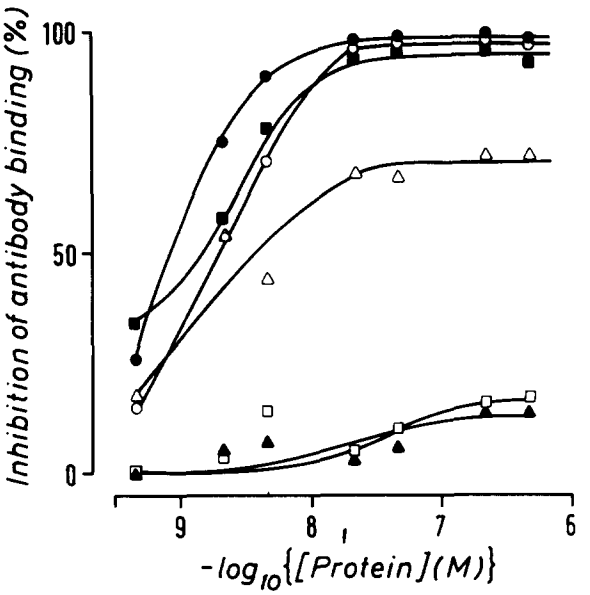

Fig. 3. ELISA with GP-I-anchored proteins. The ability of GP-I-anchored proteins to inhibit the binding of the anti-CRD antiserum to immobilised pig pAP-P was determined as described in Materials and Methods. ●, pig pDP; ○, pig pAP-P; ■, human pDP; △, sVSG; ▲, pig mDP; □, human mDP.

shown). With pAP-P and sVSG this recognition was completely abolished by prior treatment of the proteins with either 1 M HCl or nitrous acid. Whereas with human and pig pDP only nitrous acid treatment completely abolished recognition by the anti-CRD antiserum; after treatment with 1 M HCl there was still some faint recognition. Some slight recognition of human and pig mDP by the anti-CRD antiserum was observed (Fig. 2) and this was completely abolished by treatment with nitrous acid but not with 1 M HCl.

Characterisation of the epitopes involved in the CRD by ELISA

Various test antigens, G-PI-anchored proteins (Fig. 3) and individual components of the G-PI anchor (Fig. 4), were employed in a competitive ELISA system to assess their ability to inhibit the binding of the anti-CRD antiserum to immobilised pig pAP-P. Binding of the anti-CRD antiserum to immobilised pAP-P was inhibited in a concentration-dependent manner by all of the phospholipase C-solubilised G-PI anchored proteins examined (Fig. 3). Maximum percentage inhibition of binding values for pig pDP, pig pAP-P, human pDP and sVSG were 98%, 96%, 95% and 80%, respectively. Maximally 20% inhibition of binding of the anti-CRD antiserum was observed with pig or human mDP.

The ability of individual components of the G-PI anchor structure to inhibit binding of the anti-CRD antiserum to pAP-P was also examined (Fig. 4). Concentration dependent inhibition of binding of the anti-CRD antiserum to pAP-P was only observed with inositol 1,2-cyclic monophosphate, rising to a maximum of 97%. Negligible inhibition of binding (maximally 10%) was observed with inositol 1-monophosphate, inositol 2-monophosphate, inositol, glucosamine, man-

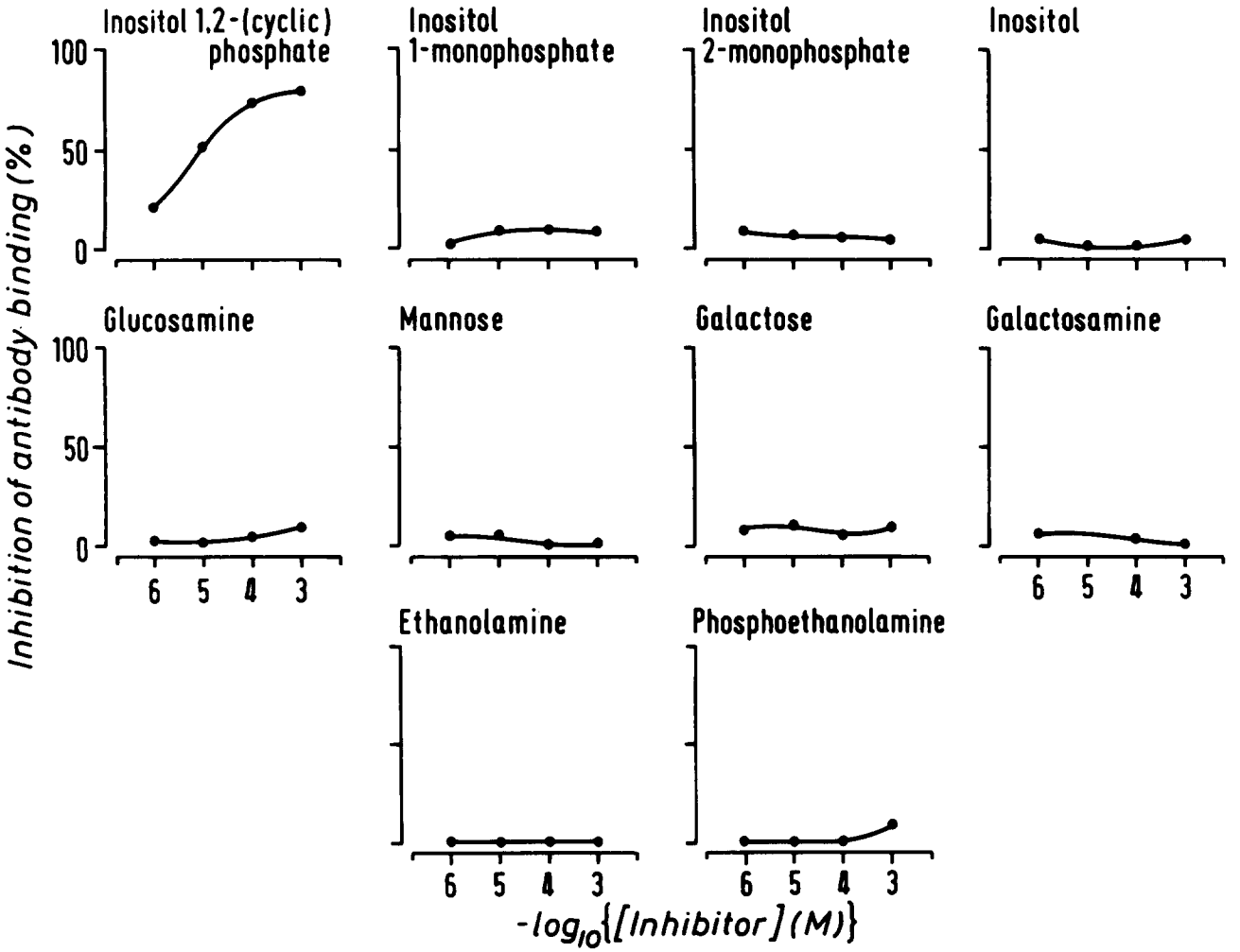

Fig. 4. ELISA with components of the G-PI-anchor. The ability of individual components of the G-PI-anchor structure to inhibit the binding of the anti-CRD antiserum to immobilised pig pAP-P was determined as described in Materials and Methods. *N*-Acetylgalactosamine and *N*-acetylneuraminic acid failed to cause any inhibition of antibody binding in the range 1 μM to 1 mM.

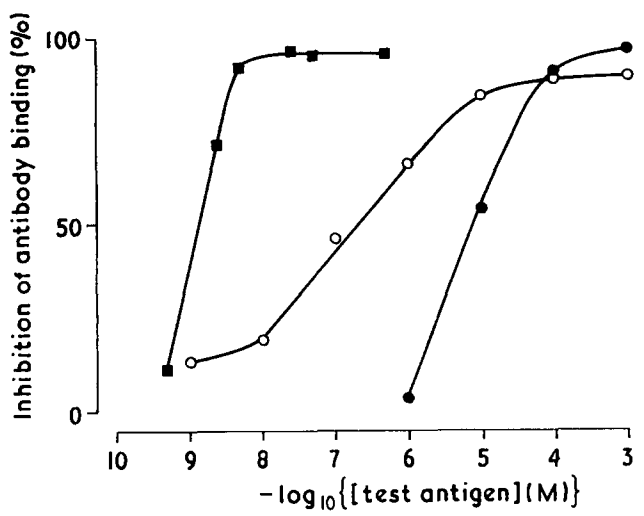

Fig. 5. ELISA with glucosaminyl-1,6-inositol 1,2-cyclic monophosphate. The ability of glucosaminyl-1,6-inositol 1,2-cyclic monophosphate (○) to inhibit binding of the anti-CRD antiserum to immobilized pig pAP-P was compared with inositol 1,2-cyclic monophosphate (●) and pig pDP (■) as described in Materials and Methods.

nose, galactose, galactosamine, *N*-acetylgalactosamine, ethanolamine, phosphoethanolamine and *N*-acetylneuraminic acid.

Recognition of an insulin-mimetic disaccharide by the anti-CRD antiserum

The competitive ELISA was used to assess the ability of the synthetic putative insulin mediator, glucosaminyl-1,6-inositol 1,2-cyclic monophosphate, to inhibit the binding of the anti-CRD antiserum to immobilised pig pAP-P (Fig. 5). This compound inhibited the binding of the anti-CRD antiserum in a dose-dependent manner in the micromolar range. The ability of all the test antigens to inhibit binding of the anti-CRD antiserum is compared, in terms of I_{50} values, in Table I. Glucosaminyl-1,6-inositol 1,2-cyclic monophosphate was 40-fold more potent in the ELISA than inositol 1,2-cyclic monophosphate, but some 100-fold less potent than any of the phospholipase C-solubilized G-PI-anchored proteins.

TABLE I

Inhibition of binding of the anti-CRD antiserum

The ability of G-PI-anchored proteins or components of the G-PI anchor to inhibit the binding of the anti-CRD antiserum to immobilized pAP-P was determined by ELISA as described in Materials and Methods. The I_{50} is the concentration of inhibitor giving 50% inhibition of antibody binding. Results are the means of duplicate determinations.

Inhibitor	I_{50}
Pig pDP	0.93 nM
Human pDP	1.47 nM
Pig pAP-P	1.92 nM
sVSG	2.52 nM
Pig mDP	> 1 μM
Human mDP	> 1 μM
Glucosaminyl-1,6-inositol 1,2-cyclic monophosphate	0.2 μM
Inositol 1,2-cyclic monophosphate	8 μM

Discussion

Membrane dipeptidase was purified from human kidney cortex following solubilization with bacterial PI-PLC by affinity chromatography on cilastatin-Sepharose [24]. A polyclonal antiserum was then raised to the purified protein. Immunoelectrophoretic blot analysis indicated that the crude antiserum recognised not only human DP but also other phospholipase C-solubilised G-PI-anchored proteins. To characterise further the epitopes involved in the cross-reactivity the population of antibodies recognising the CRD were purified by chromatography on a column of immobilized porcine pAP-P. Attempts to isolate the anti-CRD antibodies from the crude antiserum by adsorbing out those antibodies recognising the protein using the membrane form of human DP immobilized on nitrocellulose (after Ref. 8) were unsuccessful. Even after repeated incubation, the unbound fraction was still found to recognise human mDP.

Immunoelectrophoretic blot analysis showed that the anti-CRD antiserum resulting after chromatography on pAP-P-Sepharose recognised a variety of G-PI-anchored proteins that had been cleaved by phospholipase C. With porcine pAP-P and trypanosome sVSG this recognition was abolished by prior treatment of the proteins with either mild acid (1 M HCl) or nitrous acid. Mild acid treatment selectively decyclises the inositol 1,2-cyclic monophosphate, which is formed on phospholipase C cleavage of the anchor, yielding approx. 80% and 20% of inositol 1-monophosphate and inositol 2-monophosphate, respectively [30]. Nitrous acid deaminates the glucosamine residue, hydrolysing the glucosamine-inositol bond and converting the glucosamine into 2,5-anhydromannitol [30]. These results indicate that recognition of porcine pAP-P and trypanosome sVSG by the antiserum raised to human pDP is due almost entirely to cross-reactivity with the inositol 1,2-cyclic monophosphate in the G-PI anchor. In the case of human and porcine pDP, recognition by the anti-CRD antiserum was not completely abolished by mild acid treatment, only by nitrous acid treatment. Thus, on human and porcine pDP, in addition to the inositol 1,2-cyclic monophosphate epitope, there appears to be also a minor protein-specific, mild acid-insensitive, nitrous acid-sensitive epitope. As nitrous acid will deaminate any primary amine this epitope could involve either an additional phosphoethanolamine residue on the G-PI anchor, a feature which appears to be common to the G-PI anchors of higher eukaryotes [2], or a lysine residue in the polypeptide chain. The membrane forms of human and porcine DP, were not

recognized significantly by the anti-CRD antiserum on immunoelectrophoretic blot analysis, consistent with the CRD epitope being cryptic in the intact G-PI anchor and only exposed on phospholipase C cleavage.

The various G-PI-anchored proteins were also tested for their ability to inhibit the binding of the anti-CRD antiserum to immobilized pig pAP-P in a competitive ELISA. Only those proteins that had been solubilized by phospholipase C showed appreciable (80–100%) inhibition of antibody binding. The membrane forms of human and porcine DP, which possess an intact G-PI anchor, only inhibited antibody binding to a maximum of 20%. The competitive ELISA was also used to assess the ability of various individual components of the G-PI anchor to inhibit binding of the anti-CRD antiserum. Only inositol 1,2-cyclic monophosphate caused significant (97%) inhibition of binding. No such inhibition of binding was observed with inositol 1-monophosphate or inositol 2-monophosphate confirming that decyclization of the inositol 1,2-cyclic monophosphate with mild acid would abolish cross-reactivity. The lack of inhibition of binding by inositol indicates that G-PI anchored proteins cleaved by phospholipase D are unlikely to cross-react with this anti-CRD antiserum. This was confirmed by incubation of porcine mDP with partially purified plasma phospholipase D [26]. This resulted in the conversion of the amphipathic mDP to a hydrophilic form as assessed by phase separation in Triton X-114 but there was no recognition of this form of the protein by the anti-CRD antiserum on immunoelectrophoretic blot analysis (result not shown). In contrast when mDP was incubated in the presence of *Bacillus thuringiensis* PI-PLC the resulting hydrophilic form of the protein was recognized by the anti-CRD antiserum. Other components of the conserved core of the G-PI anchor (glucosamine, mannose, ethanolamine and phosphoethanolamine) and possible side-chain constituents (galactose, galactosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid) also failed to inhibit binding of the anti-CRD antiserum.

Some of the actions of insulin, nerve growth factor, interleukin-2 and other hormones appear to be mediated by inositol phosphoglycans which structurally resemble, and may be derived from, G-PI anchors [21,31–33]. If these inositol phosphoglycan mediators are generated by the action of a phospholipase C they should possess a cyclic monophosphate on the inositol residue which would be recognised by the anti-CRD antiserum. Recently, glucosaminyl-1,6-inositol 1,2-cyclic monophosphate has been synthesized and shown to stimulate lipogenesis in intact rat adipocytes in a dose-dependent manner in the micromolar range [25]. In contrast, neither of the related compounds glucosaminyl-1,6-inositol 1-monophosphate nor glucosaminyl-1,6-inositol displayed insulin-mimetic properties, indicating that the cyclic phosphate is essential for the observed biological action of glucosaminyl-1,6-inositol 1,2-cyclic monophosphate. In the present study we have shown that glucosaminyl-1,6-inositol 1,2-cyclic monophosphate is recognised by the anti-CRD antiserum and that it inhibits binding of this antiserum to immobilized pAP-P with an I_{50} in the micromolar range. The observation that glucosaminyl-1,6-inositol 1,2-cyclic monophosphate was some 40-fold more effective at inhibiting the binding of the anti-CRD antiserum than inositol 1,2-cyclic monophosphate suggests that the glucosamine moiety may contribute to the recognition by the antiserum. Three other antisera have been shown to recognise the putative inositol phosphoglycan mediators [11,22,23]. However, none of these antisera appear to recognise the inositol 1,2-cyclic monophosphate epitope. Thus, the anti-CRD antibody described in the present study may be a valuable tool for use in investigating the mechanism of action of inositol phosphoglycans in the mediation of hormone action, as well as in studies on the structure and biosynthesis of G-PI anchors.

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