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The contact site A glycoprotein of *Dictyostelium discoideum* carries a phospholipid anchor of a novel type

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The contact site A glycoprotein, a cell adhesion protein of aggregating *Dictyostelium* cells, was labeled with fatty acid, *myo*-inositol, phosphate and ethanolamine *in vivo*, indicating that the protein is anchored in the membrane by a lipid. This lipid was not susceptible to phosphatidyl inositol specific phospholipase C. When cleaved with nitrous acid or when subjected to acetolysis, the anchor released lipids which were different from those released from *Trypanosoma* variant cell surface glycoprotein, a protein with a known phosphatidyl inositol-glycan anchor. Resistance to weak and sensitivity to strong alkali indicated that the fatty acid in the contact site A glycolipid anchor was in an amide bond. On incubation with sphingomyelinase, a lipid with the chromatographic behavior of ceramide was released. These results suggest that the contact site A glycoprotein is anchored by a ceramide based lipid glycan.

Key words: cell adhesion molecule/ceramide/*Dictyostelium*/phospholipid anchor/sphingolipid

Introduction

The contact site A (csA) glycoprotein is an adhesion molecule expressed at the aggregation stage of *Dictyostelium discoideum* (Müller and Gerisch, 1978). This plasma-membrane protein is modified by two or more types of oligosaccharide chains, of which the type I chain is N-linked and heavily sulfated (Stadler *et al.*, 1983; Yoshida *et al.*, 1984; Hohmann *et al.*, 1987b). Sequence analysis of cDNA clones indicated that the csA protein is synthesized with a 19 amino acid signal peptide at the N terminus and with a hydrophobic C-terminal region (Noegel *et al.*, 1986). These are the only strongly hydrophobic regions recognized in the cDNA derived sequences, suggesting that a transmembrane region along the polypeptide chain is absent.

A hydrophobic C-terminal sequence appears to be characteristic of membrane proteins in which the primary C terminus is replaced by a phosphatidyl inositol (PI) anchor (Ferguson and Williams, 1988). Our previous studies, indicating that not only fatty acid but also *myo*-inositol is incorporated into the csA molecule (Stadler *et al.*, 1984; Stadler, 1987) suggested that this protein is modified by a typical PI anchor (Stadler *et al.*, 1988). However, final proof was not obtained since the anchor could not be cleaved by treatment of the intact protein with PI-specific phospholipase C. This result is not uncommon. Some mammalian membrane proteins that are thought to be linked to a PI anchor, such as human erythrocyte acetylcholine esterase, also proved to be highly insensitive to the phospholipase C (Roberts *et al.*, 1987). Possible reasons for this resistance are steric hindrance caused by the oligosaccharide or polypeptide moiety of the molecule, or substitutions at the inositol ring (Ferguson and Williams, 1988).

The structures of two phospholipid anchors, that of a variant cell surface glycoprotein (VSG) of *Trypanosoma brucei* (Ferguson *et al.*, 1988) and of the Thy-1 lymphocyte surface antigen (Homans *et al.*, 1988) have been elucidated. In both of these anchors, PI is linked through a non-acetylated amino sugar to an oligosaccharide chain which, at its non-reducing end, is bound by a phosphoester linkage to ethanolamine. The ethanolamine is linked through an amide bond to the α-carboxyl of the C-terminal amino acid of the protein.

In the present paper we provide evidence that the csA glycoprotein possesses a phospholipid anchor that is not PI. Our results are consistent with the view that the protein is anchored by a ceramide based lipid glycan previously unrecognized as a protein modification.

Results

Constituents of a phospholipid anchor are incorporated into the csA glycoprotein

As previously demonstrated (Stadler *et al.*, 1984), the csA protein incorporated [^{3}H]palmitic acid (Figure 1). The protein also was labeled by [^{3}H]myristic acid, although not to as great an extent as with palmitic acid (data not shown). In addition to fatty acid, label from [^{3}H]*myo*-inositol, [^{3}H]ethanolamine, or [^{32}P]phosphate was incorporated into a band at the 80 kd position of the csA glycoprotein (Figure 1). The csA glycoprotein was the only membrane protein which incorporated substantial label from *myo*-inositol and from ethanolamine, when cells were labeled during the early stages of development. After 6 months of exposure of an autoradiograph, faint labeling with inositol was also detected in a 130 kd protein. Similarly, weak labeling of a 130 kd band was seen after incubation of cells with [^{3}H]palmitic acid or [^{3}H]ethanolamine (Figure 1). The labeled 130 kd protein is probably identical with a glycoprotein which, like the csA glycoprotein, is highly sulfated and expressed during early development (Stadler *et al.*, 1983).

In order to confirm that label was located in the csA molecule, the glycoprotein was affinity purified by the use of a monoclonal antibody directed against its polypeptide moiety (Figure 2A–C). Non-covalently bound lipids were removed by extraction of acidified aqueous dispersions of the protein with chloroform/methanol. As shown in Figure 2C, [^{3}H]palmitic acid label was still associated

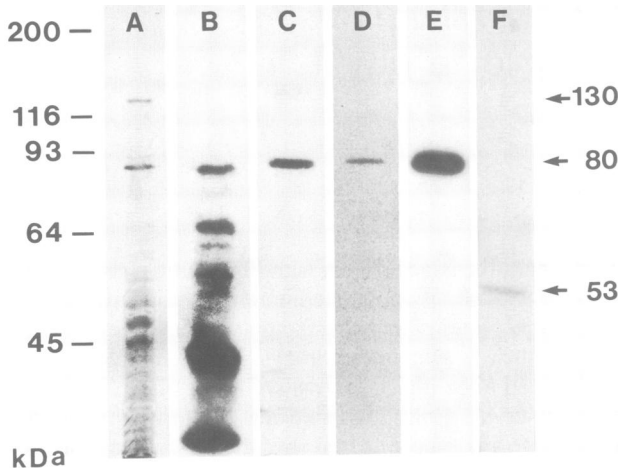

Fig. 1. Labeling of a 80 kd membrane protein in *D.discoideum* cells by **A**, [^{3}H]palmitic acid, **B**, [^{32}P]phosphate, **C**, [^{3}H]ethanolamine, **D**, [^{3}H]*myo*-inositol, **E**, anti-csA antibody, and **F**, labeling of a 53 kd protein by [^{3}H]*myo*-inositol in tunicamycin treated cells. Cells were labeled during the first 6 h of development. For A–E, 5×10^6 cell equivalents of particulate fractions were loaded per lane; for F, particulate fraction was extracted with 1% octyl-oligo-oxyethylene, and 2×10^7 cell equivalents of the insoluble material were loaded per lane. The fluorograph of D was exposed for 10 days, that of A,C,F for 3 days, B,E, overnight.

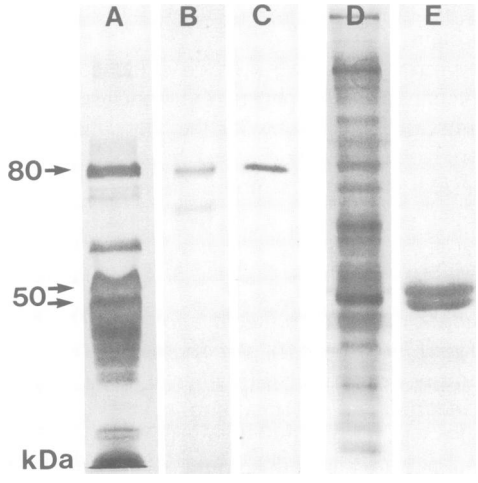

Fig. 2. A–C, affinity purification of [^{3}H]palmitic acid labeled csA glycoprotein, and **D** and **E**, of an N-terminal csA fragment released from mutant cells defective in type 2 glycosylation. A–C, developing AX2 wild type cells were *in vivo* labeled for 6 h with [^{3}H]palmitic acid and the particulate fraction of the cells was extracted with butanol/water. The water phase was chromatographed on Q-Sepharose, and the csA glycoprotein affinity purified on an mAb 41-71-21 column eluted with 4 M $MgCl_2$. Proteins of the csA containing fractions of A, the Q-Sepharose column and B, of the affinity purified material were analyzed by SDS–PAGE and silver staining; C, proteins of the affinity purified material were also blotted and fluorographed for [^{3}H]palmitic acid. D and E, extracellular medium was harvested from the modB mutant strain HG220 and chromatographed on Q-Sepharose. Fractions containing csA were subjected to affinity purification using an anti-csA antibody column, and the affinity purified csA fragments were eluted at pH 12. D, proteins in the Q-Sepharose fraction and E, the affinity purified fraction were analyzed by SDS–PAGE and silver staining. Proteins obtained from 3×10^7 cells were applied to lanes A and D, and from 2×10^8 cells to lanes B, C and E.

with the affinity purified glycoprotein. Similar results were obtained with material labeled by [^{3}H]ethanolamine or [^{32}P]phosphate. [^{3}H]Inositol labeling was not tested with the affinity purified material, but incorporation of the label into the csA protein was demonstrated by incubating cells with [^{3}H]*myo*-inositol in the presence of tunicamycin. With this drug, attachment of type 1 and type 2 oligosaccharide chains to the protein can be prevented, which results in reduction of the apparent molecular mass of the final product by 27 kd (Hohmann *et al.*, 1987b). The [^{3}H]*myo*-inositol label in tunicamycin treated cells was incorporated into the 53 kd csA protein produced under these conditions (Figure 1F), which confirmed that the label was associated with csA, and showed that modification of the protein with an inositol derivative was independent of type 1 and type 2 glycosylation.

It has been reported that phosphate is linked to serine residues in the csA glycoprotein (Schmidt and Loomis, 1982). We subjected the affinity purified [^{32}P]phosphate labeled glycoprotein to total hydrolysis of the polypeptide. No significant label was found in phosphoserine, indicating that little, if any, [^{32}P]phosphate was linked directly to the polypeptide backbone of the glycoprotein.

The lipid is associated with the C-terminal region of the csA glycoprotein

Hohmann *et al.* (1987a) found that in modB mutants which are defective in type 2 glycosylation, a 68 kd precursor of the csA molecule is transported to the cell surface. This precursor is highly susceptible to a *D.discoideum* protease, giving rise to two fragments with apparent molecular masses of ~50 kd which are released into the medium (Figure 2D–E). These fragments are glycosylated by sulfated type 1 oligosaccharide (Hohmann *et al.*, 1987a). As judged from their apparent molecular masses, these fragments comprise ~70% of the polypeptide moiety of the csA glycoprotein. Sequencing of the affinity purified fragments revealed that the first 12 amino acids of the N terminus of the mature glycoprotein were present in the fragment, indicating that the protein is associated with the plasma membrane only at its C-terminal region. In order to examine whether [^{3}H]palmitic acid is incorporated into the csA fragments released into the medium, cells of wild-type AX2 and of the HG220 modB mutant strain were incubated in parallel with [^{3}H]palmitic acid. Blots of proteins from either a membrane fraction of wild-type cells or from the medium of a mutant culture were first subjected to fluorography for [^{3}H]palmitic acid, and subsequently to labeling of the csA polypeptide moiety with antibody. Although the antibody recognized roughly equal amounts of csA protein in wild-type membranes and mutant medium, the ^{3}H-label of palmitic acid was only detected in the membrane-bound glycoprotein (Figure 3).

PI-specific phospholipases C do not cleave the csA anchor; nitrous acid removes the anchor

The foregoing labeling studies gave results that one would expect if the csA protein is anchored by PI. Typical PI anchors can be cleaved by PI-specific phospholipase C from *Staphylococcus aureus* (Ferguson *et al.*, 1985b), that of VSG also by a phospholipase C-like hydrolase from *T.brucei* (Bülow and Overath, 1986). The compound removed by phospholipase C is diacylglycerol. Another characteristic of typical PI anchors is their release by nitrous acid. This sensitivity to deamination is due to the presence of a non-acetylated amino sugar that links the inositol residue to the oligosaccharide chain and thus to the protein (Ferguson

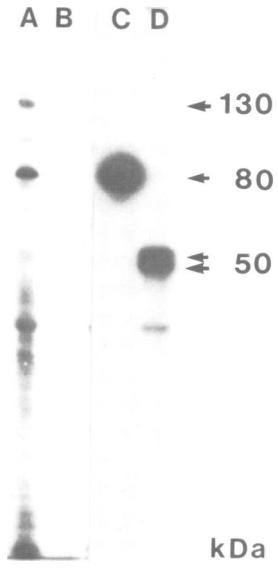

Fig. 3. Absence of fatty acid label from the N-terminal csA fragment released into the medium from mutant HG220 cells. **A** and **C**, cells of wild type AX2 or **B** and **D**, the HG220 mutant strain were labeled with [^{3}H]palmitic acid. A and C, proteins of the particulate fraction from AX2 cells or B and D, from HG220 culture medium were separated by SDS−PAGE, blotted onto nitrocellulose, fluorographed for [^{3}H]palmitic acid (A and B), and subsequently labeled with anti-csA antibody for autoradiography (C and D). Proteins obtained from 200 μl cell suspension, which contained 2 × 10^6 cells, were applied per lane.

and Williams, 1988; Strang *et al.*, 1986). The product liberated by nitrous acid is PI.

The results obtained by phospholipase C treatment of the [^{3}H]palmitic acid labeled csA glycoprotein are summarized in Figure 4. Neither the enzyme from *S.aureus* nor that from *T.brucei* detectably reduced the label associated with the protein (Figure 4, A−C). Under the same conditions at least 98% of the [^{3}H]myristic acid label of VSG from *T.brucei* was removed from its protein moiety by both enzymes. Since VSG incorporates only myristic acid (Ferguson *et al.*, 1985a; Gurnett *et al.*, 1986), the possibility remained that in the csA glycoprotein only myristic acid was incorporated into the PI-anchor, while palmitic acid was otherwise linked to the protein. To exclude this, the experiment was repeated with csA from cells that were labeled with [^{3}H]myristic acid. Again, no loss of label from the csA glycoprotein was observed after incubation with either of the two phospholipases (data not shown). Resistance of the csA anchor to *S.aureus* phospholipase C was confirmed by lipid extraction with organic solvent. No [^{3}H]palmitic acid labeled compounds could be detected after thin-layer chromatography (TLC) of the csA extract (Figure 4D), while from VSG that was treated with phospholipase C under identical conditions, labeled material with the mobility of diacylglycerol was extracted (Figure 4E).

In order to examine whether the phospholipid anchor can be removed specifically by deamination, the glycoprotein was treated with nitrous acid and subjected to SDS−PAGE (Figure 5, A−F). Fluorography showed no recovery of [^{3}H]palmitic acid label at the 80 kd position of the csA glycoprotein. However, antibody labeling also was not observed and no protein band was detected by silver staining, indicating that the protein was degraded or altered by deamination.

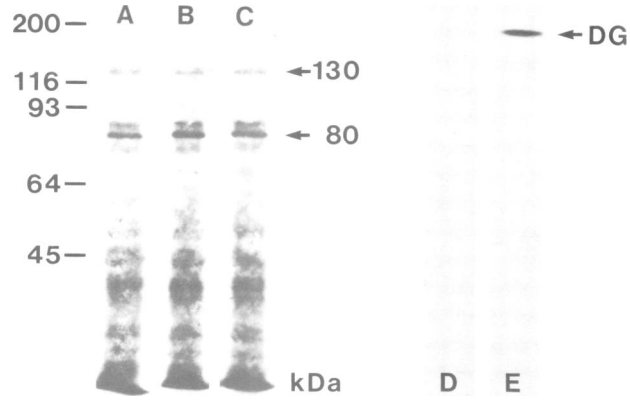

Fig. 4. Resistance of the fatty acid label of csA to removal by phospholipase C. **A−C**; A, *in vivo* [^{3}H]palmitic acid labeled and detergent solubilized membrane proteins were incubated with phospholipase C from *S.aureus*, B, with phospholipase C-like hydrolase from *T.brucei* or C, without enzyme. The incubated samples were subjected to SDS−PAGE, blotting onto nitrocellulose, and fluorography. **D** and **E**; D, affinity purified, [^{3}H]palmitic acid labeled csA or E, purified *T.brucei* VSG were incubated in parallel with *S.aureus* phospholipase C, and extracted with diethyl ether. The extract was subjected to TLC with development in the polar solvent system B (see Materials and methods), and to fluorography. In this solvent system diacylglycerol and fatty acids have similar mobilities.

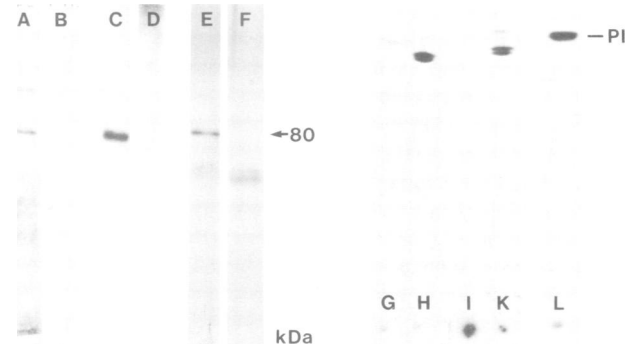

Fig. 5. Effects of nitrous acid treatment on the csA glycoprotein, and analysis of the lipids released from csA and VSG. **A−F**; A, C and E, [^{3}H]palmitic acid labeled and affinity purified csA glycoprotein was incubated at pH 4.0 without or B, D and F, with nitrous acid and subjected to SDS−PAGE. For A and B, the material was blotted onto nitrocellulose and fluorographed for ^{3}H-label; for C and D, blots were labeled with anti-csA antibody; for E and F, the gel was silver stained. **G−K**; G and H, [^{3}H]palmitic acid or I and K, [^{32}P]phosphate labeled, affinity purified csA glycoprotein was incubated at pH 4.0, G and I, without or H and K, with nitrous acid. The material was acidified, extracted with chloroform/methanol, subjected to TLC with development in the polar solvent system B and to fluorography. **L**, [^{3}H]myristic acid labeled, purified *T.brucei* VSG was incubated with nitrous acid and subsequently treated in parallel with H and K. The position of unlabeled PI standard is indicated.

Nitrous acid failed to produce lipid-free but otherwise intact protein, so release of the anchor was assayed by extraction of nitrous acid treated and untreated glycoprotein with organic solvent. Only after nitrous acid treatment could material labeled with [^{3}H]palmitic acid or [^{32}P]phosphate be extracted. The major labeled spot showed a lower mobility in polar solvent on thin-layer plates than the PI cleaved from VSG (Figure 5, G−L).

The results described above do not rule out the presence of a PI anchor. The phospholipases C might be hindered by the protein moiety, or by the structure of the oligosaccharide chain that links the anchor to the protein, and

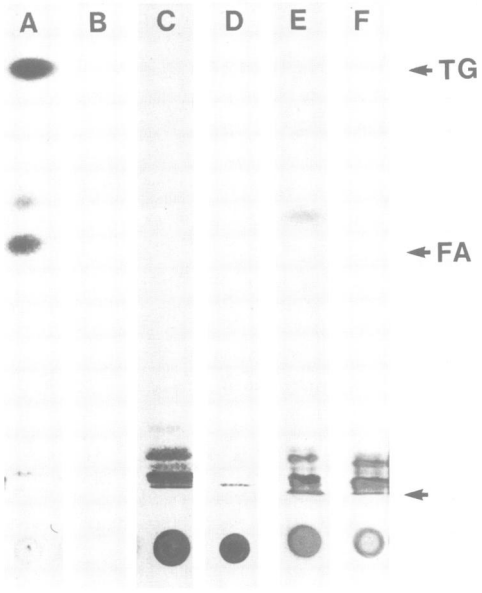

Fig. 6. Products of acetolysis of the csA and VSG phospholipids separated by TLC in a non-polar solvent system. **A**, [^{3}H]myristic acid labeled products from purified *T.brucei* VSG showed high mobility in solvent A (see Materials and methods). The plates were fluorographed. Positions of authentic, unlabeled diacylglycerol acetate (TG) and palmitic acid (FA) are indicated on the right (myristic acid migrated similarly to palmitic acid in this system). **B** and **C**, [^{3}H]palmitic acid labeled products from csA, B, before and C, after acetolysis, run in parallel to A. The products from csA showed little mobility, indicating that they were more polar than the VSG products. No free palmitic acid was detected, suggesting absence of a fatty acid ester linkage in the csA anchor. **D–F**, products from the free, nitrous acid removed csA anchor, D, before and E and F, after acetolysis. The csA glycoprotein had been labeled either D and E, with [^{3}H]palmitic acid or F, with [^{32}P]phosphate. TLC of D–F was run in parallel with A–C to show that the same products of acetolysis were obtained from the free anchor as from the entire protein. For all treatments the csA glycoprotein had been affinity purified and extracted under acidic conditions with chloroform/methanol. The arrow indicates the boundary between the pre-concentration zone and the analytical layer.

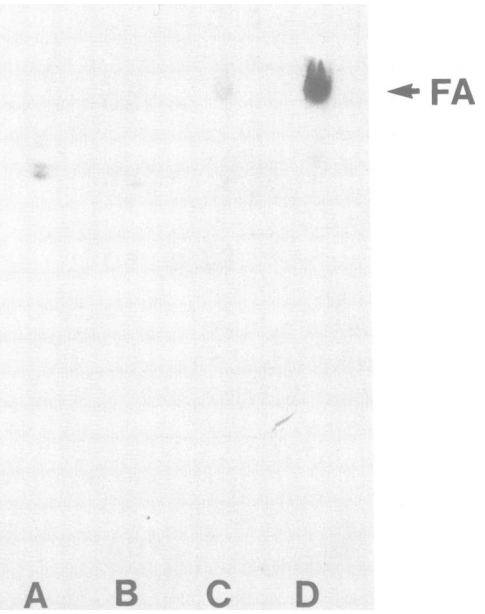

Fig. 7. Resistance of the csA anchor to hydroxylamine and mild alkali treatment, and release of fatty acid under strong alkaline conditions. [^{3}H]Palmitic acid labeled csA glycoprotein was purified as for Figure 6, and lipid released by nitrous acid was extracted. The extract was either **A**, untreated or **B**, incubated with neutral hydroxylamine, **C**, treated for 1 h with 0.6 N methanolic KOH at room temperature, or **D**, for 18 h with 1.0 N methanolic KOH at 70°C. The material was acidified, extracted with hexane/diethyl ether, and the extract analyzed by TLC using the non-polar solvent system A. Labeled fatty acid was liberated only under the strong alkaline conditions of D, by which amide bonds are cleaved. The position of palmitic acid (FA) standard is indicated on the right. Fatty acid hydroxamates had a mobility similar to palmitic acid in this solvent system.

the compounds produced by nitrous acid treatment of csA and VSG might differ only in fatty acid composition or in substitutions at the inositol residue. In order to prove that the csA phospholipid is not PI, the material extracted from [^{3}H]palmitic acid labeled and nitrous acid treated csA was incubated with *S.aureus* phospholipase C. Again, no diacylglycerol was generated when this free phospholipid was exposed to the phospholipase (data not shown).

Acetolysis of the csA phospholipid and treatment with hydroxylamine and alkali indicate that the anchor does not consist of PI

Acetolysis of [^{3}H]myristate labeled VSG resulted in release of three lipids which were separated by TLC (Figure 6). One of the major products co-migrated with diacylglycerol acetate, i.e. it migrated as triacylglycerol, and another co-migrated with free fatty acid. The identity of a third product, which was only weakly labeled and migrated between fatty acid and triacylglycerol, is unknown. Acetolysis of [^{3}H]palmitate or ^{32}P-labeled csA released lipids which showed little mobility in a non-polar solvent system (Figure 6). No diacylglycerol acetate or free fatty acid, the products obtained by acetolysis of PI, were obtained from the csA anchor.

Lipid released by nitrous acid from [^{3}H]palmitic acid labeled csA was treated with hydroxylamine. This treatment did not result in release of label in the form of fatty acid hydroxamates (Figure 7). This result suggested that the fatty acid is not bound in an ester linkage. When the nitrous acid released material was treated with alkali under mild conditions, only a small amount of the label was liberated as free [^{3}H]palmitic acid. Alkali treatment under stronger conditions, as used for release of fatty acid from an amide bond, did result in near quantitative release of the label as fatty acid (Figure 7). Taken together, these results suggest that fatty acid is bound in the lipid anchor of csA in an amide, rather than in an ester or thioester, bond. This opens the possibility that the lipid anchor of csA is an inositol ceramide.

Cleavage by sphingomyelinase suggests a ceramide based anchor

Lipid released from [^{3}H]palmitic acid labeled csA by nitrous acid was incubated with sphingomyelinase from human placenta. This enzyme preparation converted the lipid into a labeled compound which migrated with ceramide on thin-layer plates (Figure 8). Conversion was not quantitative, even after 12 h incubation, probably because the lipid is not an optimum substrate for the enzyme. Sphingomyelinase from *S.aureus* also converted nitrous acid released lipid from csA into a compound migrating with ceramide on TLC plates, but at a rate lower than that of the enzyme from placenta (not shown). CsA protein was degraded on

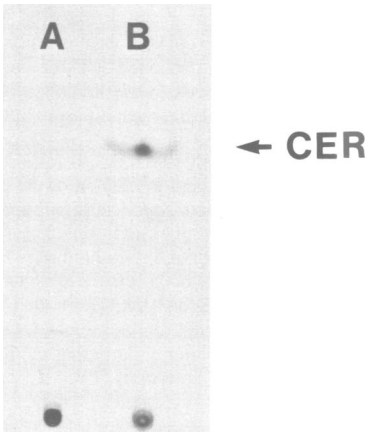

Fig. 8. Susceptibility of the csA lipid to sphingomyelinase. [^{3}H]Palmitic acid labeled csA glycoprotein was purified, cleaved by nitrous acid, and the anchor extracted as for Figure 7. The lipid was subjected to TLC with development in chloroform/methanol, 9:1, after incubation **A**, without or **B**, with sphingomyelinase from human placenta. The position of an unlabeled ceramide standard (CER) is indicated on the right.

incubation with commercial sphingomyelinases, probably due to contaminating proteases.

Discussion

Is the lipid anchor a specific feature of the csA glycoprotein related to its function in cell adhesion?

After labeling of cells with *myo*-inositol during the pre-aggregation phase, the label was preferentially incorporated into the csA glycoprotein. The only other protein detectably labeled under our conditions was a 130 kd glycoprotein that might be another cell adhesion protein. A similar selectivity for the csA glycoprotein was observed upon labeling with ethanolamine (Figure 1).

Sadeghi *et al.* (1988) have reported that yet another developmentally regulated membrane glycoprotein of *D. discoideum*, antigen 117, incorporates fatty acid, *myo*-inositol and ethanolamine. In the gel system used by these authors, antigen 117 formed bands at the 69 and 72 kd positions. We could not detect any inositol and fatty acid labeled protein other than the csA glycoprotein in the 60–80 kd molecular mass region by SDS–PAGE of membrane proteins from aggregating cells. This result raises the question whether antigen 117 as characterized by Sadeghi *et al.* (1987) is identical with the csA glycoprotein. Sequencing of antigen 117 would clarify that point.

The high selectivity of csA labeling we observed suggests a function of the lipid anchor in the activity of csA in cell adhesion. The csA glycoprotein of *D.discoideum* mediates specifically the EDTA-resistant ('Ca-independent') type of intercellular adhesion that is characteristic for cells in the aggregation stage. This EDTA-stable adhesion allows cells in agitated suspension, where they are continuously dissociated by shear force, to form large clumps. In mutants that lack the csA protein, the EDTA-stable adhesiveness as it occurs in suspension is drastically reduced (Noegel *et al.*, 1985). However, mutant cells are still capable of aggregating on an agar surface where they move slowly together without being affected by shear. These results indicate that the csA glycoprotein is specialized for rapidly glueing colliding cells together and that other adhesion molecules are sufficient for aggregation under conditions that prevent the cells from being rapidly separated after having made contact. For the Thy-1 antigen and the decay accelerating factor of complement in blood cells, it has been shown that the phospholipid anchor allows fast lateral diffusion of the protein in the plasma membrane (Woda and Gilman, 1983; Ishihira *et al.*, 1987). It can be assumed, therefore, that the lipid anchor confers high membrane mobility to the csA glycoprotein, as it is required for rapidly matching interacting molecules on an adjacent cell surface. The neuronal cell adhesion molecule, N-CAM, exists in two versions, N-CAM$_{120}$ with a truncated C terminus to which a phospholipid anchor is attached, and another form with a hydrophobic transmembrane amino acid sequence (He *et al.*, 1986). These differences might be reflected in the rates of cell association mediated by the two versions of the protein.

Constituents and suggested structure of the csA phospholipid anchor

The results presented here show that the phospholipid anchor of the csA glycoprotein does not consist of PI, although fatty acid, phosphate, *myo*-inositol and ethanolamine are incorporated into the csA glycoprotein. These are the constituents of VSG of *T.brucei* and other membrane proteins, which are anchored by an ethanolamine linked PI glycan (Ferguson and Williams, 1988).

Evidence against a PI lipid in csA is provided by the following results, (i) the lipid released from [^{3}H]palmitic acid labeled csA glycoprotein with nitrous acid behaved differently in TLC in two solvent systems than did the PI that was released from VSG by nitrous acid; (ii) the lipid released by nitrous acid was not susceptible to hydrolysis by PI-specific phospholipase C that liberates diacylglycerol from the VSG anchor; (iii) acetolysis of csA did not result in release of diacylglycerol acetate, showing that a phosphoglyceride moiety is not present in the lipid anchor. The products of acetolysis were more polar than diacylglycerol acetates (Figure 6A and C). They were *in-vivo* labeled not only by [^{3}H]palmitic acid but also by [^{32}P]phosphate (Figure 6, E–F). (iv) Labeled palmitic acid was not released from the csA anchor by hydroxylamine or mild alkali treatment, indicating that the fatty acid was not linked to glycerol by an ester bond. Only under alkaline conditions that allow the cleavage of amide bonds was fatty acid almost completely released.

In ceramides the fatty acids are amide linked. Ceramides are common constituents of sphingomyelins from which they can be released by sphingomyelinases. We have therefore tested whether the csA anchor is susceptible to various sphingomyelinases. One of them, sphingomyelinase from human placenta, released from csA a fatty acid labeled lipid with the chromatographic properties of ceramide. Although these results suggest that the csA anchor is a sphingolipid in which a ceramide is linked to inositol, structural proof needs to be obtained.

The PI anchors of VSG and Thy-1 antigen are conjugated to the C terminus of the protein through an oligosaccharide chain. The oligosaccharide is linked at one end to the inositol through an amino sugar that is susceptible to deamination by nitrous acid, and at the other end to the protein through an ethanolamine residue. In addition to the bridging ethanolamine, some PI glycan anchors contain a further

ethanolamine residue with a free amino group (Ferguson and Williams, 1988). Our results are consistent with a similar lipid–protein linker in the csA glycoprotein, but caution must be exercised in interpretation of the data. Although ethanolamine is incorporated with high selectivity into the csA glycoprotein *in vivo*, this incorporation is not strictly indicative of a lipid anchor. Tisdale and Tartakoff (1988) have reported the covalent addition of ethanolamine to a hydrophilic, cytosolic protein in a number of mammalian cell lines. Release of the csA anchor by nitrous acid suggests that the lipid is linked to glucosamine, the only amino sugar present in the csA molecule (Müller *et al.*, 1979), but lack of recovery of the protein after nitrous acid treatment indicates that other bonds also are affected.

If one assumes the lipid anchor to be connected by an oligosaccharide chain to the protein, it would follow that the csA protein is associated with at least three types of carbohydrate chains. A product that lacks both type 1 and type 2 carbohydrate is produced in the presence of tunicamycin, which was found to incorporate label from *myo*-inositol (Figure 1). This result compares well with the findings of Bangs *et al.* (1985) and Ferguson *et al.* (1986) who showed that PI-glycan tailing occurs independently of N-glycosylation.

Possible relationships of the csA anchor to other lipid structures

Various yeasts, mycobacteria and green plants have been shown to contain ceramide-linked glycans, at least some of which have a ceramide-phosphate-inositol-glycan structure (Lee and Ballou, 1965; Carter *et al.*, 1969; Hsieh *et al.*, 1978; Kaul and Lester, 1978). A ceramide glycan has also been identified in *D.discoideum* (Wilhelms *et al.*, 1974). The structures of these ceramide glycans are consistent with the labeling characteristics of the csA lipid anchor. Our results are in accord with the possibility that the csA molecule is attached through an ethanolamine residue to a glycan-inositol-phosphate-ceramide of the type structurally characterized by Carter *et al.* (1969), Lee and Ballou (1965) and Hsieh *et al.* (1978). However, structural characteristics of the csA anchor remain to be elucidated.

The resistance of the csA lipid to PI-specific phospholipase C is shared by some other lipid anchored proteins (Low, 1987). In the examples which have been reported, it has been assumed that structural peculiarities render the PI-anchors of these proteins resistant to PI-phospholipase C hydrolysis. Roberts *et al.* (1988) have reported that inositol in the lipid anchor of human erythrocyte acetylcholinesterase is acylated with palmitic acid and suggested that this acylation confers resistance to PI-specific phospholipase C. It is possible that the csA anchor of *D.discoideum* is the prototype of another class of protein linked lipid structures. These structures also might be present in some lipid anchored mammalian proteins which proved to be resistant to PI-specific phospholipases. Susceptibility to sphingomyelinases would establish a similarity between the lipids of these proteins and the lipid by which the csA glycoprotein of *D.discoideum* is anchored.

Materials and methods

Cell culture and in vivo labeling

Cells of the axenically growing AX2-214 strain of *D.discoideum* and of the modB mutant strain HG220 (Hohmann *et al.*, 1987a) were cultivated at 23°C in nutrient medium to a density of not more than 5×10^6 cells/ml (Malchow *et al.*, 1972). Development was initiated by washing cells in 17 mM Soerensen phosphate buffer, pH 6.0, and resuspending them in the buffer at 1×10^7 cells/ml. After continued agitation on a rotary shaker with 150 r.p.m. at 23°C, AX2 cells were harvested at 6–8 h in the aggregation competent stage; culture medium from HG220 cells was harvested at 20–22 h. For inhibition of N-glycosylation, 0.4 μg/ml of tunicamycin (Boehringer Mannheim) was added to suspensions of AX2 cells at the beginning of development, and expression of the csA protein was stimulated for 12 h by cAMP pulses of 20 nM amplitude applied every 6 min (Ochiai *et al.*, 1982). Radiolabeled compounds were added at the beginning of development in phosphate buffer at an activity of 1–2 mCi per 10 ml cell suspension. Compounds used for *in vivo* labeling were [^{3}H]palmitic acid (New England Nuclear, NET 043, 28 Ci/mmol), [^{3}H]myristic acid (Amersham, TRK 797, 41 Ci/mmol), [^{3}H]*myo*-inositol (Amersham, TRK 883, 110 Ci/mmol), [^{3}H]ethanolamine (Amersham, TRK 462, 30 Ci/mmol) and [^{32}P]orthophosphate (Amersham, PBS 11, carrier-free).

Protein purification

For purification of the csA glycoprotein, particulate fractions from AX2 cells were obtained by freezing and thawing, and centrifugation for 15 min at 10 000 *g*. The pellet was extracted with butanol/water (Müller *et al.*, 1979), the aqueous phase was made to 0.1% Triton X-100, dialyzed against 10 mM piperazine–HCl, pH 5.5, and applied to a Q-Sepharose column (Pharmacia). CsA glycoprotein was eluted in a NaCl gradient, identified by dot blotting (Stadler *et al.*, 1982) and applied to an affinity column prepared by crosslinking 21 mg of IgG of mAB 41-71-21 to 1 ml of Protein A–Sepharose (Pharmacia) with 20 mM dimethylpimelimidate (Pierce Chemical Company) (Schneider *et al.*, 1982). mAb 41-71-21 recognizes the csA protein moiety (Bozzaro and Merkl, 1985). After application, the column was washed at 4°C with 20 mM Hepes Buffer, pH 6.5, containing 2.5 mM EDTA and 1% Triton X-100, to remove unbound proteins, and csA glycoprotein was eluted with 4.5 M $MgCl_2$ in the Hepes–EDTA buffer without Triton X-100. Fractions containing csA glycoprotein were dialyzed against distilled water, and the protein was concentrated by precipitation with 9 vols of acetone pre-cooled to −20°C. Similar results were obtained by washing the column with 50 mM sodium phosphate, pH 11, containing 0.1% Triton X-100, elution of the csA glycoprotein with 50 mM sodium phosphate, pH 12, without the detergent, and immediate neutralization of the 0.5 ml fractions with 0.2 ml of 0.5 M acetic acid, similar to the method of Bülow and Overath (1986).

CsA fragments released from HG220 cells were isolated from medium after removal of cells at 1500 *g*, centrifugation of the supernatant for 20 min at 10 000 *g* and passage of the supernatant through a 0.45 μm Millipore filter. After addition of Triton X-100 to 0.1%, the filtrate was adjusted to pH 5.5 with HCl and chromatographed on Q-Sepharose. CsA fragments were then purified by affinity chromatography and elution at pH 12 as described above.

As an authentic control for PI-glycan anchors, VSG from *T.brucei* MiTat 1.4 was carried through procedures used to establish the lipid nature of the csA anchor. Purified VSG labeled with [^{3}H]myristic acid was the gift of Dr R.Bülow, Max-Planck-Institut für Biologie, Tübingen, FRG.

Gel electrophoresis, fluorography and immunoblotting

Proteins were separated by SDS–PAGE in 10% acrylamide gels according to Laemmli (1970), and either silver stained (Oakley *et al.*, 1980) or blotted onto BA85 (Schleicher and Schuell) nitrocellulose (Vaessen *et al.*, 1981). For detection of ^{3}H-label, blots were dipped into 20% (w/v) 2,5-diphenyloxazole in toluene, dried and exposed to Kodak XAR-5 film at −70°C. For subsequent immunolabeling, the nitrocellulose was washed with toluene and incubated with [^{125}I]mAb 33-294-17, which recognizes the polypeptide moiety of SDS denatured csA (Bertholdt *et al.*, 1985).

Release of lipid anchors with nitrous acid

CsA glycoprotein eluted from affinity columns with $MgCl_2$ contained non-covalently bound lipids. These lipids were removed by extracting aqueous protein suspensions made to 0.1 N with HCl twice with chloroform/methanol (2:1, v/v). The acidification was necessary for removal of these lipids. CsA or VSG was treated with nitrous acid according to Ferguson *et al.* (1985a) and the released lipid extracted after acidification with chloroform/methanol as above. For TLC, the extract was spotted onto 20 × 20 cm plates coated with silica gel G and with a pre-concentration zone (Merck). The plates were developed first in acetone/petroleum ether, 1:3 (v/v), and then after drying, in chloroform/methanol/water/acetic acid, 25:15:4:2 (v/v) ('solvent system B').

Incubation with enzymes

For treatment with phospholipase C, either detergent solubilized particulate

fraction, purified csA glycoprotein or VSG were suspended in 300 μl of 50 mM Tris–acetate, pH 7.4, with 0.1% detergent, and incubated for 1 h at 37°C with 20 μg of a PI-specific phospholipase C preparation from *S.aureus* (a gift of Dr M.Low, Columbia University, New York) or with phospholipase C-like hydrolase from *T.brucei* (Bülow and Overath, 1986). The detergent was sodium cholate for the *S.aureus* and Triton X-100 for the *T.brucei* enzyme. After incubation, the mixtures were subjected to SDS–PAGE (Figure 4A–C). Alternatively, lipids were extracted with diethyl ether and examined by TLC on silica gel G plates with solvent system B (Figure 4D and E).

For treatment with sphingomyelinases, lipids released from [^{3}H]palmitic acid labeled csA with nitrous acid were incubated with sphingomyelinase from human placenta (Sigma catalogue no. S1380) or from *S.aureus* (Sigma catalogue no. S8633) essentially according to Morrison (1969). A solution of about 5 μg lipid in chloroform was mixed with 10 μg Triton X-100, the solvent was evaporated and the residue dissolved in a mixture of 20 μl chloroform and 300 μl diethyl ether. To this solution was added 40 μl of buffer (1 M sodium acetate, pH 5.2, for placenta enzyme; 1 M Tris–acetate, pH 7.6, for *S.aureus* enzyme), 20 μl of 0.4 M $MgCl_2$ and 1 unit of enzyme. Tubes were sealed and incubated under constant mixing at 37°C for 12 h. Organic solvent was evaporated with nitrogen, liberated ceramide was extracted with diethyl ether and the extract examined on HPTLC plates (Sigma) which were developed in chloroform/methanol, 9:1 (v/v).

Acetolysis, treatment with hydroxylamine and alkaline hydrolysis

Acetolysis of csA, VSG, or of lipid released from csA by nitrous acid, was performed according to Ferguson *et al.* (1985a,b). The liberated lipid was extracted with chloroform/methanol, 2:1 (v/v), and examined by TLC on silica gel G plates. The plates were developed in petroleum ether/diethyl ether/acetic acid, 80:20:1 (v/v) ('solvent A').

Material released by nitrous acid from [^{3}H]palmitic acid labeled csA was subjected to treatment with methanolic hydroxylamine (Heusser, 1968), to mild saponification with 0.6 N KOH in 90% methanol/chloroform, 1:1 (v/v) for 1 h at room temperature (Dawson, 1960), or to more rigorous saponification with 1 N KOH in ~80% methanol for 18 h at 70°C (Morrison, 1969). Following these treatments, the reaction mixtures were acidified with HCl, liberated fatty acids or hydroxamates were extracted with hexane/diethyl ether, 1:1 (v/v), and the extracts applied onto 10 × 10 cm HPTLC silica gel plates (Sigma) which were developed in solvent system B.

For detection of ^{3}H-label, plates were sprayed with Enhance (New England Nuclear) and exposed to Kodak XAR-5 film at −70°C. For detection of unlabeled standards, plates were sprayed with 0.03% diphenylhexatriene in chloroform (Sigma) and examined under UV light.

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