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Cleavage with phospholipase of the lipid anchor in the cell adhesion molecule, csA, from *Dictyostelium discoideum*

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Abstract

A cell adhesion molecule, 80-kDa csA, is involved in EDTA-resistant cell contact at the aggregation stage of *Dictyostelium discoideum*. A 31-kDa csA was isolated from the 80-kDa csA by treatment with *Achromobacter* protease I. Results from thin-layer chromatography and MALDI-TOF MS analysis indicated that the 31-kDa csA contains ceramide as a component of glycosylphosphatidyl-inositol (GPI). Comparison between the 80-kDa csA and the 31-kDa csA treated with phosphatidylinositol-specific phospholipase C (PI-PLC) or GPI-specific phospholipase D (GPI-PLD) was carried out. Our results indicated that the GPI-anchor of the 31-kDa csA was more sensitive to PI-PLC treatment than that of the 80-kDa csA, and that the anchor in both was easily cleaved by GPI-PLD treatment. They suggested that the resistance of 80-kDa csA to PI-PLC treatment was due to steric hindrance and myo-inositol modification. The results of the 80-kDa csA and the 31-kDa csA treated with sphingomyelinase were similar to those with PI-PLC treatment. In the presence of 1,10-phenanthroline, a GPI-PLD inhibitor, development of *Dictyostelium* was markedly inhibited, suggesting that GPI-PLD is functional in developmental regulation through cell adhesion.

Keywords: GPI-anchor; PI-PLC; GPI-PLD; Cell adhesion molecule; csA; 1,10-phenanthroline; *Dictyostelium*

1. Introduction

The cell adhesion molecule, csA, is involved in EDTA-resistant cell contact at the aggregation stage of *Dictyostelium discoideum*. CsA is a glycoprotein with an apparent molecular mass of 80 kDa and is anchored to the cell membrane by means of a glycosylphosphatidyl-inositol (GPI)-linkage with ceramide, although GPI-anchors generally consist of diacylglycerol or alkyl-acylglycerol. Stadler et al. (1989) reported that the GPI-anchor region in csA glycoprotein was not cleaved by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), although the GPI-anchor region was found to be sensitive to treatment with an endogenous PI-PLC enzyme (da Silva and Klein, 1991).

Since a common mode of membrane attachment via the GPI-anchor was first clarified (Ferguson and Williams, 1988), over one hundred membrane proteins have been found to be anchored to the membrane through a GPI-linkage. Phosphoinositides are released from GPI-anchors by attack of PI-PLC. This has led to the idea that the cleavage of GPI-anchors might be involved in receptor-mediated triggering reactions (Low, 1989). However, the role of GPI-anchors in signal transduction remains unclear.

The resistance of the GPI-anchor cleavage in csA to PI-PLC treatment (Stadler et al., 1989) suggested that modification of the inositol ring by fatty acids conferred resistance to PI-PLC treatment as observed in human acetylcholinesterase (Roberts et al., 1988), or that the structure of the GPI-anchor with ceramide instead of diacylglycerol conferred resistance to PI-PLC treatment. In this study, comparison between PI-PLC and GPI-specific phospholipase D (PLD) treatment was carried out to clarify the resistance to cleavage the GPI-anchor in csA by PI-PLC treatment. Moreover, the involvement of GPI-PLD in *Dictyostelium* development will also be discussed.

2. Materials and methods

2.1. Purification of 80-kDa csA and 31-kDa csA

Cells of *D. discoideum* AX2-214 were cultivated at 22 °C in nutrient medium with 1.8% maltose as described by Watts and

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Ashworth (1970). Development was begun by washing the cells in 17 mM Sorensen's phosphate buffer, pH 6.1 (standard buffer), and continued for 8 h to develop aggregation-competent cells with shaking at 150 rpm at 22 °C. Purified 80-kDa csA was prepared as a single band by silver staining as described previously (Yoshida, 1987). Particulate fractions from aggregation-competent cells were prepared by freezing and thawing, and by centrifugation for 20 min at 10,000 $\times g$. Plasma membrane-enriched fractions were obtained from the particulate fractions by dextran 500/polyethylene glycol 6000 separation. The plasma membrane-enriched fractions were incubated with ca. 40% (v/v, a final conc.) of butanol to extract the 80-kDa csA. The butanol/water extract was subjected to DEAE-cellulose (DE-52) chromatography. The fractions containing the 80-kDa csA were pooled, suspended in Laemmli buffer, heated for 5 min and applied to a preparative electrophoresis system (Biophoresis III, Atto, Tokyo, Japan). The samples were eluted with 0.37 M Tris–HCl buffer, pH 8.8, containing 5% glycerol and collected in fractions of 0.5 mL. The 80-kDa csA showed a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the standard method (Laemmli, 1970) and silver staining. Fractions containing the 80-kDa csA were pooled and precipitated with acetone, and the precipitates were dried under reduced pressure. Next, to isolate the 31-kDa csA glycopeptide (Yoshida et al., 1997b), ca. 100 μg of purified 80-kDa csA was suspended in 200 μl of 0.1% SDS. Then, *Achromobacter* protease I (Wako Pure Chemicals, Osaka, Japan), which is highly specific for lysine (Tsunasawa et al., 1989), was added until the enzyme to substrate ratio (w/w) was 1/100 to 1/500. The reaction was carried out at 37 °C for 16 h in 0.01 M phosphate buffer, pH 7.8. The products were suspended in Laemmli buffer, and heated for 5 min. They were applied to a preparative electrophoresis system, and fractions of 0.5 mL were collected. The fractions containing the 31-kDa csA were detected by dot tests using horseradish peroxidase-conjugated wheat germ agglutinin (WGA, Sigma, Missouri, USA) and SDS-PAGE. Fractions including the 31-kDa csA were collected and precipitated by addition of acetone as reported previously (Yoshida et al., 1997a).

2.2. Treatment of csA with PI-PLC, sphingomyelinase or GPI-PLD

The acetone-dried samples of the 80-kDa csA and the 31-kDa csA were suspended in 10 μl of 0.1% sodium deoxycholate and treatment with PI-PLC from *Bacillius cereus* (a final conc. of 40 U/mL, Sigma) was carried out at 37 °C for 2 or 20 h in 0.05 M Tris–HCl buffer, pH 7.5. Alternatively, PI-PLC treatment of the 31-kDa csA was carried out at 37 °C for 4 h in the presence of protease inhibitors (Protease inhibitor set, Boehringer-Mannheim, Mannheim, Germany). Moreover, 0.5 μL of the 31-kDa csA treated with PI-PLC for 4 h was added to 0.5 μL of 30 mg/mL a-cyano-4-hydroxycinnamic acid dissolved in methanol–water (1:1, v/v). The sample was allowed to air-dry at room temperature and then subjected to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS. Mass analysis was performed on a Voyager-DE™ PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA) in the linear mode using a nitrogen laser (337 nm) and an acceleration voltage of 23.5 kV. The 80-kDa csA and the 31-kDa csA treated with sphingomyelinase from human placenta (a final conc. of 55 U/mL, Sigma) in 10 μL of acetate buffer (pH 5.5) including 20 mM $MgCl_2$ at 37 °C for 20 h. In the case of the GPI-PLD, the acetone-dried samples of the 80-kDa csA and the 31-kDa csA were suspended in 10 μL of 0.05 M Tris–HCl buffer, pH 8.0, and treatment with the GPI-PLD (a final conc. of 2 U/μL, a kind gift from Dr. Brodbeck, University Bern), which was purified from bovine serum as described in the paper of Hoener and Brodbeck (1992), was carried out at 37 °C for 2 or 20 h. Each reaction was stopped by addition of Laemmli buffer for SDS-PAGE. The samples were subjected to SDS-PAGE, and Western blotting was carried out according to the method of Towbin et al. (1979). The nitrocellulose filters (BA 85, Schleicher and Schuell, Dassel, Germany) were incubated with horseradish peroxidase-conjugated WGA or with monoclonal antibody 80L5C4 against csA (a kind gift from Dr. Siu, University of Toronto). The filters were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Organon Teknica, Belgium). After incubation with a probe, a filter was washed four times by changing washing buffer, 10 mM Tris–HCl, pH 7.4, 0.05% Tween 20, 150 mM NaCl. The validity of PI-PLC, sphingomyelinase, or GPI-PLD in the GPI-anchor cleavage of the 80-kDa csA and 31-kDa csA was determined by a shift to the lower molecular mass of each csA molecule on SDS-PAGE.

2.3. Preparation of lipid components from the GPI-anchor

Samples treated with PI-PLC were extracted three times with chloroform. The chloroform extracts were removed by the processes of centrifugation, pooling, and evaporation under reduced pressure. The sample was kept overnight in a desiccator and resolved using TLC. TLC was developed in chloroform/methanol (9:1, v/v). TLC plates were sprayed with orcinol/H_2SO_4, and heated at 110 °C.

2.4. Assay of cell agglutination and chemotaxis

Cell agglutination was measured by a modification of the method of Beug and Gerisch (1973). Cells were starved for 8 h without or with 1–100 μM 1,10-phenanthroline (a final conc.), an inhibitor of GPI-PLD, washed with standard buffer, adjusted to 1×10^7/mL, and rotated at 40 rpm for 20 min at 22 °C in the presence or absence of 10 mM EDTA. Cells were counted with a hemocytometer under a light microscope. Single cells and doublets were scored as unaggregated cells. Cells were spread on agar plates containing 1–100 μM 1,10-phenanthroline (final conc.). For the assay of chemotaxis toward cyclic AMP, cells were starved for 8 h, washed with standard buffer, and transferred to the Teflon surface of a Petriperm dish (Heraeus, Hanau, Germany). Micropipettes filled with 10^{-3} M cyclic AMP solution were used as described by Gerisch and Keller (1981).

3. Results and discussion

3.1. Properties of a 31-kDa csA

A 31-kDa cell adhesion molecule, csA, was reported previously to be derived from an 80-kDa csA by *Achromobacter* protease I treatment (Yoshida et al., 1997b). Thin-layer chromatography (TLC) of the 31-kDa csA treated with phosphatidylinositol-specific phospholipase C (PI-PLC) was conducted to determine whether the 31-kDa csA contained a glycosylphosphatidyl-inositol (GPI)-anchor component. Three spots appeared on the TLC plate (Fig. 1A). The middle spot was identified as ceramide, but the middle spot did not appear in the case of the 31-kDa csA without PI-PLC (data not shown). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS analysis was conducted to clarify the size of a released ceramide. Though the 31-kDa csA showed the molecular mass of 31 kDa on SDS-PAGE, a signal from the 31-kDa csA without PI-PLC was m/z 10,297.68 and a signal from the 31-kDa csA treated with PI-PLC was m/z 9338.84 on MALDI-TOF MS (Fig. 1C and D). The molecular mass of the ceramide released by PI-PLC treatment was determined to be

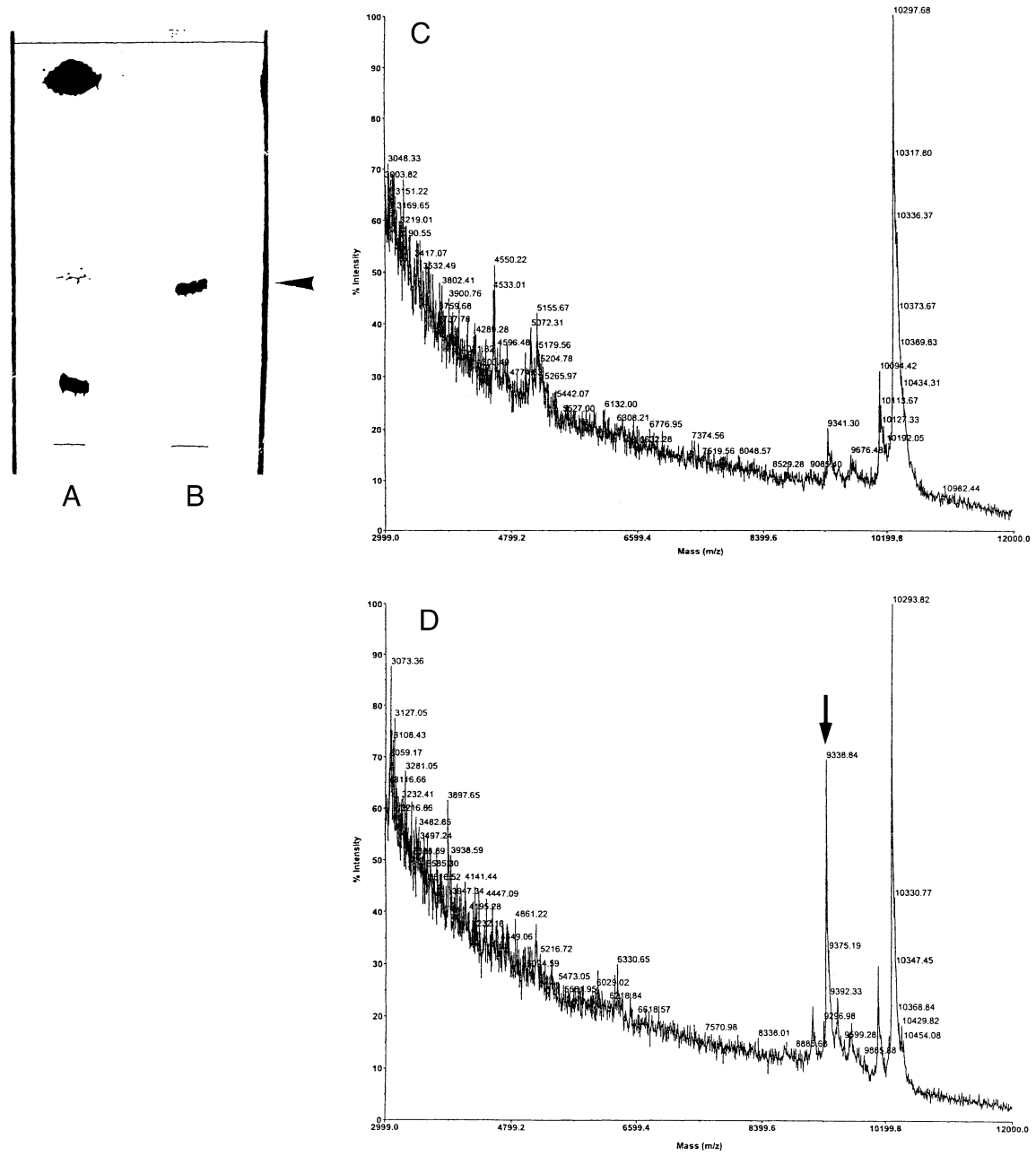

Fig. 1. Identification of lipid components in the 31-kDa csA by thin-layer chromatography and MALDI-TOF MS analysis. In A and B, thin-layer chromatography was conducted. A: Chloroform extract of the 31-kDa csA treated with 40 U/mL (a final conc.) of PI-PLC at 37 °C for 20 h. B: Ceramide standard. Development and staining were carried out as described in Materials and methods. An arrowhead shows the position of ceramide released by PI-PLC treatment. In C and D, MALDI-TOF MS analysis was conducted. C: 31-kDa csA without PI-PLC treatment. D: 31-kDa csA treated with 40 U/mL (a final conc.) of PI-PLC at 37 °C for 4 h. An arrow shows a signal from the 31-kDa csA treated with PI-PLC. Abscissa: mass spectrum, mass (m/z); ordinate: relative abundance, intensity (%).

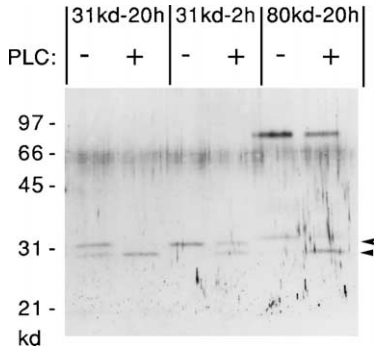

Fig. 2. Treatment of the 80-kDa csA and 31-kDa csA with PI-PLC. The 80-kDa csA and 31-kDa csA were treated with PI-PLC for 2 or 20 h. The samples were separated by SDS-PAGE. The gels were blotted onto nitrocellulose filters and incubated with peroxidase-conjugated WGA. Arrowheads indicate the positions of the 31-kDa csA and 29-kDa csA. Positions of molecular mass markers are indicated on the left. 31kd−20 h, 31kd−2 h and 80kd−20 h indicate PI-PLC treatment of 31-kDa csA for 20 h, 31-kDa csA for 2 h, and 80-kDa csA for 20 h, respectively.

958.84 by subtraction. When diacylglycerol was applied on TLC under the same condition, a signal appeared in the uppermost position (data not shown). These reconfirmed the fact that the GPI-anchor consisted of ceramide instead of diacylglycerol (Stadler et al., 1989). Ceramide-based anchors have been found in *Saccharomyces cerevisiae* and *Trypanosoma cruzi* (Conzelmann et al., 1992; de Lederkremer et al., 1990). The 31-kDa csA retains a GPI-anchor as well as the clustered O-linked carbohydrates, which mainly react with wheat germ agglutinin (WGA) (Yoshida et al., 1997b). The composition of the uppermost spot on the TLC plate is identified as hydrocarbons of C_{16}–C_{33} by gas chromatography-mass spectroscopy as described previously (Yoshida et al., 1997a), while those of the lower spot are deoxycholate. These results suggest that hydrocarbons interact with the GPI-anchor region of csA glycoprotein through hydrophobic bonds. It is unlikely that these hydrocarbons are derived from fatty acids. Treatment to release hydrocarbons from fatty acids was not applied in the preparation stage before the sample was subjected to TLC. There is evidence to suggest that GPI-anchor proteins interact with cholesterol in the caveolae which is involved in endocytosis (Simons and Ikonen, 1997). In *D. discoideum*, hydrocarbons might exist instead of cholesterol and interact with GPI-anchor proteins.

3.2. Treatment of 80-kDa csA and 31-kDa csA with PI-PLC or sphingomyelinase

The 80-kDa csA and 31-kDa csA were incubated with PI-PLC as described in Materials and methods. The 31-kDa csA was highly susceptible to hydrolysis with 40 U/mL (a final conc.) of PI-PLC at 37 °C for 20 h, as demonstrated by the lower molecular mass shift from 31 to 29 kDa on SDS-PAGE. In contrast, more than 50% of the 80-kDa csA remained amphiphilic after PI-PLC treatment under the same conditions (Fig. 2). Incubation of the 31-kDa csA with PI-PLC at 40 U/mL (a final conc.) for 2 h was not sufficient for complete lower molecular mass shift of the 31-kDa csA. The differences in sensitivity to PI-PLC treatment between the 31-kDa csA and 80-kDa csA might have been due to the relative ease with which PI-PLC can gain access to the cleavage site in the GPI-anchor region of each molecule. Although the 31-kDa csA could be converted completely with PI-PLC, the product was not recognized by an antibody (Glyko Biomedical, CA, USA) against the cross-reacting determinant (CRD), which is a common carbohydrate epitope exposed on PI-PLC cleavage (Zamze et al., 1988). The antibody showed the reactivity with acetylcholinesterase from bovine erythrocytes (Wako Pure Chemicals) treated with PI-PLC (data not shown), indicating the adequacy of the antibody in the reaction with CRD.

The csA retains a GPI-anchor with ceramide as well as the glycoproteins of *S. cerevisiae* and *T. cruzi* whose GPI-anchor structures have been studied in depth (Conzelmann et al., 1992; de Lederkremer et al., 1990). Sphingomyelinase cleavage was thought to be effective on the GPI-anchor with ceramide because ceramide is a common constituent of sphingomyelin that can be released by sphingomyelinase treatment. Treatment of the 80-kDa csA and 31-kDa csA with sphingomyelinase as described in Materials and methods reduced the molecular mass in both csA as those results observed with PI-PLC treatment (Fig. 3). Treatment of 80-kDa csA with sphingomyelinase led to a strong signal of 31-kDa csA and a weak signal of 40-kDa, 29-kDa csA. It was conceivable that 29-kDa csA was a product from 31-kDa csA by sphingomyelinase treatment, and that 31-kDa, 40-kDa csA were a degradation product of the 80-kDa csA during treatment.

The validity of PI-PLC or sphingomyelinase in the GPI-anchor cleavage of the 80-kDa csA or the 31-kDa csA was determined by a shift to a lower molecular mass of each csA molecule on SDS-PAGE. In this method, however, it is

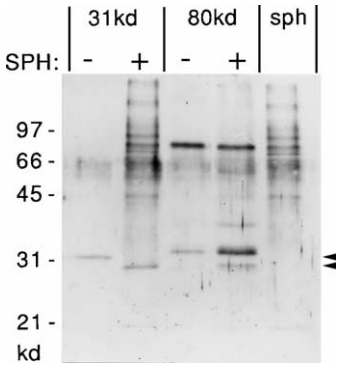

Fig. 3. Treatment of the 80-kDa csA and 31-kDa csA with sphingomyelinase. The 80-kDa csA and 31-kDa csA were treated with sphingomyelinase for 20 h. The samples were separated by SDS-PAGE. The gels were blotted onto nitrocellulose filters and incubated with peroxidase-conjugated WGA. Arrowheads indicate the positions of the 31-kDa csA and 29-kDa csA. Positions of molecular mass markers are indicated on the left. 31 kDa−, 31-kDa csA incubated without sphingomyelinase; 31 kDa+, 31-kDa csA incubated with sphingomyelinase; 80 kDa−, 80-kDa csA incubated without sphingomyelinase; 80 kDa+, 80-kDa csA incubated with sphingomyelinase; sph, sphingomyelinase was incubated alone. In 80 kDa− and 80 kDa+, half of the reaction products was applied on each lane, whereas in the other lanes the whole reaction products were applied. Sphingomyelinase shows the reactivity with WGA.

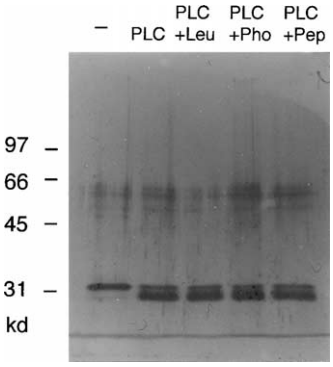

Fig. 4. Effects of PI-PLC in the presence of protease inhibitors. PI-PLC treatment of the 31-kDa csA was carried out at 37 °C for 4 h in the presence of each protease inhibitor. SDS-PAGE and staining were carried out as described in Fig. 2. –, without PI-PLC and protease inhibitors; PLC, with PI-PLC and without protease inhibitors; PLC+Leu, with PI-PLC and leupeptin; PLC+Pho, with PI-PLC and phosphoramidon; PLC+Pep, with PI-PLC and pepstatin.

possible that protease contaminants in PI-PLC or sphingomyelinase enzyme sources may have been responsible for the molecular mass shift during incubation. To investigate this possibility, the 31-kDa csA was incubated with PI-PLC at 40 U/mL (a final conc.) at 37 °C for 4 h in the presence of each protease inhibitor: leupeptin (serine protease inhibitor, a final conc. of 4.8 μg/mL); phosphoramidon (metallo endopeptidase inhibitor, a final conc. of 194 μg/mL); pepstatin (aspartate protease inhibitor, a final conc. of 4.8 μg/mL) (Fig. 4). In the case of the 80-kDa csA, the same results were observed (data not shown). In addition to these protease inhibitors, antipain dihydrochloride (an inhibitor of papain, trypsin and cathepsin A, B, a final conc. of 200 μg/mL); bestatin (aminopeptidase inhibitor, a final conc. of 50 μg/mL); aprotinin (serine protease inhibitor, a final conc. of 10 μg/mL) were used. However, the effects of PI-PLC treatment were still observed in the presence of these protease inhibitors. These results confirmed that the molecular mass shifts of 31-kDa csA was due to GPI-anchor cleavage by either PI-PLC or sphingomyelinase, and not digestion by protease contaminants in each enzyme source.

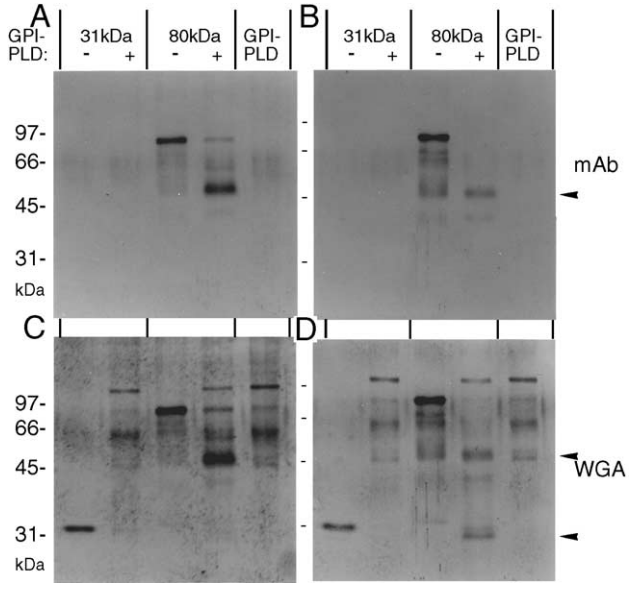

Fig. 5. Treatment of the 80-kDa csA and 31-kDa csA with GPI-PLD. The 80-kDa csA and 31-kDa csA were treated with GPI-PLD for 2 h (panel A, C) or 20 h (panel B, D). The samples were separated by SDS-PAGE. The gels were blotted onto nitrocellulose filters, incubated with monoclonal antibody 80L5C4 against csA. The filters were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (panel A, B). These filters were next incubated with peroxidase-conjugated WGA (panel C, D). 31 kDa–, 31-kDa csA incubated without GPI-PLD; 31 kDa+, 31-kDa csA incubated with GPI-PLD; 80 kDa–, 80-kDa csA incubated without GPI-PLD; 80 kDa+, 80-kDa csA incubated with GPI-PLD; GPI-PLD, GPI-PLD was incubated alone. GPI-PLD shows the reactivity with WGA. A major band is GPI-PLD with an apparent molecular mass of 100 kDa and a few lower molecular mass bands than 100 kDa are degradation products of GPI-PLD. Arrowheads indicate the positions of the 51-kDa csA and 29-kDa csA.

3.3. Treatment of the 80-kDa csA or 31-kDa csA with GPI-PLD

It was possible that modification of the inositol ring with fatty acids in the GPI-anchor or the structure of the GPI-anchor with ceramide instead of diacylglycerol might have been responsible for the resistance of 80-kDa csA to PI-PLC treatment. However, the GPI-anchor with ceramides in *S. cerevisiae* and *T. cruzi* can

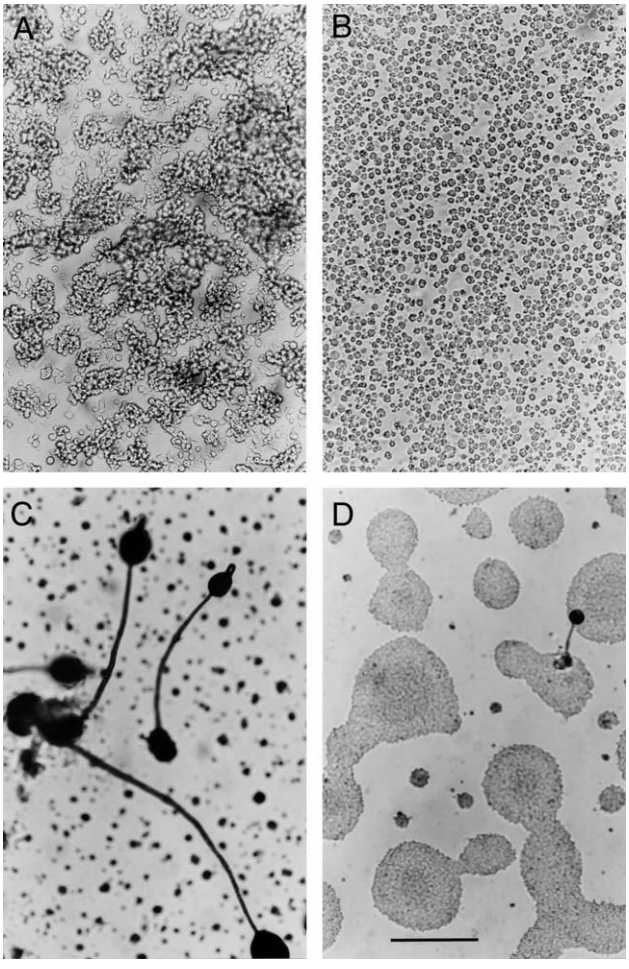

Fig. 6. Photographs of cell agglutination and morphogenesis on agar plates. Assay of cell agglutination in the presence of EDTA was carried out without (A) or with 100 μM 1,10-phenanthroline (B) as described in Materials and methods. *D. discoideum* morphogenesis was observed on agar plates without (C) or with 100 μM 1,10-phenanthroline (D) Scale bar indicates 400 μm.

be cleaved with PI-PLC, showing that ceramides in GPI-anchor do not confer the resistance to PI-PLC treatment (Conzelmann et al., 1992; de Lederkremer et al., 1990). GPI-PLD treatment of the 80-kDa csA and the 31-kDa csA was carried out to investigate the effects of modification of the inositol ring with fatty acids, because GPI-PLD activity is not affected by myo-inositol acylation (Deeg and Davitz, 1995). Therefore, the 80-kDa csA and 31-kDa csA were incubated separately with GPI-PLD at 2 U/μl (a final conc.) at 37 °C for 20 h. The GPI-anchors in both 80-kDa csA and 31-kDa csA were clearly susceptible to hydrolysis with GPI-PLD (Fig. 5). In the case of the 80-kDa csA, two glycopeptides with molecular masses of 51-kDa and 29-kDa were observed: a 51-kDa csA that reacted with the monoclonal antibody 80L5C4 against csA and with WGA, and a 29-kDa csA that reacted with only WGA. The monoclonal antibody 80L5C4 recognizes a region of the NH_2 terminus between Val-123 and Glu-172 of the 80-kDa csA (Kamboj and Siu, 1988), and WGA mainly reacts with clustered O-linked carbohydrates located in the COOH terminus of the 80-kDa csA (Yoshida et al., 1997b). It was possible that both 51-kDa csA and 29-kDa csA might have been produced from the 80-kDa csA by protease contaminants in the GPI-PLD enzyme source. However, the molecular mass shift was observed irrespective of the presence of protease inhibitors in the GPI-PLD treatment as well as PI-PLC treatment in the presence of protease inhibitors. GPI-PLD treatment of the 80-kDa csA for 2 h led to only 51-kDa csA, whereas treatment of 80-kDa csA for 20 h yielded the 51-kDa csA and 29-kDa csA (Fig. 5). These results suggested that the 51-kDa csA was an intermediate product, and that the 29-kDa csA was the final product of GPI-PLD treatment. On the other hand, GPI-PLD treatment of the 31-kDa csA led to the disappearance of the band, suggesting that the antigens recognized by WGA disappeared during GPI-PLD treatment. Taken together with the results of PI-PLC and GPI-PLD treatment, they suggest that 29-kDa csA was the final product of PI-PLC, sphingomyelinase or GPI-PLD treatment, and that the resistance of 80-kDa csA to PI-PLC treatment is due to steric hindrance and myo-inositol modification.

3.4. Influence of a GPI-PLD inhibitor on cell adhesion, chemotaxis and development

The sensitivity of the 80-kDa csA to GPI-PLD treatment suggested that in *Dictyostelium* the GPI-anchored proteins including the 80-kDa csA might be cleaved by an endogenous GPI-PLD in vivo (Metz et al., 1994; Lierheimer et al., 1997). To investigate the possible function of GPI-PLD in *Dictyostelium* cell adhesion and development, *Dictyostelium* cells were allowed to develop for 8 h in the presence of 1,10-phenanthroline, an inhibitor of GPI-PLD, after beginning starvation. In the presence of 100 μM 1,10-phenanthroline (a final conc.), EDTA-resistant cell contact as well as EDTA-sensitive cell contact were markedly inhibited (Fig. 6B, Table 1). Cells allowed to develop in the absence of 1,10-phenanthroline retained EDTA-resistant and EDTA-sensitive cell contacts (Fig. 6A). On the other hand, cells treated with 100 μM 1,10-phenanthroline showed sufficient chemotaxis toward cyclic AMP. This suggested that 1,10-phenanthroline used in experiments was not poisonous in cell viability. When cells were allowed to develop on agar plates containing 100 μM 1,10-phenanthroline (a final conc.), most of the cells remained as loose aggregates and they did not show any morphogenesis after this aggregation. Only a few tiny fruiting bodies were observed (Fig. 6D). Normal development was observed on agar plates without 1,10-phenanthroline (Fig. 6C). These results suggested that GPI-PLD might be functional in cleavage of GPI-anchored proteins during development and be involved in developmental regulation through cell adhesion.

Table 1
Effects of 1,10-phenanthroline on cell agglutination

(Single cells %)

		0 μM	1 μM	10 μM	100 μM
+EDTA	Range	17–36	16–31	48–63	90–100
	Mean	23	24	56	94
−EDTA	Range	18–32	16–28	12–31	51–76
	Mean	26	21	23	60

Cells were allowed to develop for 8 h without or with 1–100 μM 1,10-phenanthroline, washed, and rotated at 40 rpm for 20 min at 22 °C. The mean and range were derived from three separate experiments. +EDTA, EDTA-resistant cell contact; −EDTA, EDTA-sensitive cell contact.

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