近畿大農総研報 9：89～95（2001）
Bull. Inst. Compr. Agr. Sci. Kinki Univ. 9：89～95（2001）

Properties of Lipid Anchor in Cell Adhesion Molecule, csA, from *Dictyostelium discoideum*

Motonobu YOSHIDA*,**

Synopsis

A cell adhesion molecule, csA, is a glycoprotein with a molecular weight of 80-kDa and is involved in EDTA-resistant cell contact at the aggregation stage of *Dictyostelium discoideum*. A 31-kDa csA with glycosylphosphatidyl-inositol (GPI) anchor was isolated from a 80-kDa csA by treatment of *Achromobacter* protease I. Comparison between the 31-kDa csA and the 80-kDa csA on treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) or phospholipase D (PLD) was carried out. The results indicated that the GPI-anchor in the 31-kDa csA was more sensitive to PI-PLC treatment than that in the 80-kDa csA, and that the anchor in both was easily cleaved by PLD treatment. In the presence of 1,10-phenanthroline, a PLD inhibitor, development of *Dictyostelium* was markedly inhibited, suggesting that PLD is functional in developmental regulation.

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 weight of 80 kDa and is anchored to the cell membrane by means of a glycosylphosphatidyl-inositol (GPI)-linkage with ceramide instead of the diacylglycerol, which is a general component of GPI-anchor[1]. Stadler *et al* [1]. 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 source.

Since a common mode of membrane attachment via the GPI-anchor was first clarified[2], 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[3]. However, the role of GPI-anchors in signal transduction remains unclear.

The resistance of the GPI-anchor cleavage in csA to PI-PLC treatment[1] suggested that modification of the inositol ring by fatty acids conferred resistance to PI-PLC treatment as observed in human acetylcholine receptor[5] 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 phospholipase D (PLD) treatment was carried out to clarify the resistance to cleavage of the GPI-anchor in csA by PI-PLC treatment, and the possible involvement of PLD was investigated in *Dictyostelium* development.

* Department of Agriculture, Faculty of Agriculture, Kinki University, Nakamachi, Nara 631-8505, Japan (近畿大学農学部農学科)
** Institute for Comprehensive Agricultural Sciences, Kinki University, Nakamachi, Nara 631-8505, Japan (近畿大学農学総合研究所)

Materials and Methods

Purification of 80-kDa csA and 31-kDa csA. Cells of *D. discoideum* AX2-214 were cultivated at 22℃ in nutrient medium with 1.8% maltose as described by Watts and Ashworth[5]. 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℃. Purified 80-kDa csA was prepared to be shown a single band by a silver staining as described previously[6]. Particulate fractions from aggregation-competent cells were prepared by freezing and thawing, and by centrifugation for 20 min at 10,000 x 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 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 sample buffer, heated for 5 min and applied to a preparative electrophoresis system (Biophoresis III, Atto). 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) 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[7], ca. 100 μg of purified 80-kDa csA was suspended in 200 μl of 0.1% SDS. Then, *Achromobacter* protease I (Wako Chemicals), which is highly specific for lysine[8], was added until the enzyme to substrate ratio (w/w) was 1/100 to 1/500. The reaction was carried out at 37℃ for 16 h in 0.01M phosphate buffer, pH 7.8. The products were suspended in sample 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 peroxidase-conjugated WGA and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions including the 31-kDa csA were collected and precipitated by addition of acetone as reported in previously[9].

Treatment of csA with PI-PLC or PLD The acetone-dried samples of the 31-kDa csA or the 80-kDa csA were suspended in 10 μl of 0.1% sodium deoxycholate and reaction with PI-PLC from *Bacillius cereus* (a final conc. of 40 units ml^{-1}, Sigma) was carried out at 37℃ for 2 h or 20 h in 0.05 M Tris-HCl buffer, pH 7.5. Alternatively, PI-PLC treatment was carried out in the presence of protease inhibitors (Protease inhibitor set, Boehringer Mannheim). The acetone-dried samples of the 31-kDa csA or the 80-kDa csA were suspended in 10 μl of 0.05 M Tris-HCl buffer, pH 8.0, and reaction with PLD (a final conc. of 3 units μl^{-1}, Sigma) was carried out at 37℃ for 2 h or 20 h. Each reaction was stopped by addition of a sample 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*[10]. The nitrocellulose filters (BA 85, Schleicher and Schuell) were incubated with horseradish peroxidase-conjugated WGA (Sigma). The validity of PI-PLC or PLD in the GPI-anchor cleavage of the 80-kDa csA or 31-kDa csA was determined by the lower molecular weight shift of each csA molecule on SDS-PAGE.

Assay of cell agglutination and chemotaxis Cell agglutination was measured by a modification of the method of Beug *et al*[11]. Cells were starved for 8 h, washed with standard buffer, adjusted to 1 x 10^7 ml^{-1}, and rotated at 40 rpm for 20 min at 22℃ 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. 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). Micropipettes filled with 10^{-3} M cyclic AMP solution were used as described by Gerisch and Keller[12].

Results and discussion

Treatment of 80-kDa csA and 31-kDa csA with PI-PLC The 80-kDa csA and 31-kDa csA were incubated with phosphatidylinositol-specific phospholipase C (PI-PLC) as described in Materials and Methods. The 31-kDa csA was completely susceptible to treatment with 40 units ml^{-1} of PI-PLC (a final conc.) at 37℃ for 20 h, as demonstrated by the lower molecular weight 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 (Figure 1). Incubation with PI-PLC at 40 units ml^{-1} (a final conc.)

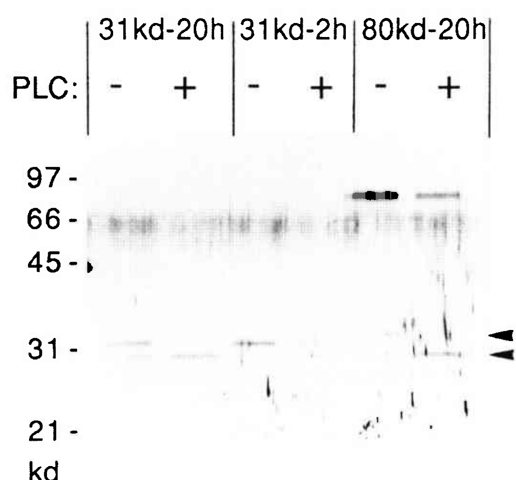

Figure 1. Treatment of the 31-kDa or 80-kDa csA with PI-PLC.
The 31-kDa csA or 80-kDa csA was treated with PI-PLC for 2 h or 20 h. The samples were separated by SDS-PAGE. The gels were blotted onto nitrocellulose filters and incubated with peroxidase-conjugated WGA. Arrowheads indicate the positions of the 31-kDa csA and 29-kDa csA (hydrophilic type). Positions of molecular weight markers are indicated on the left. 31kd-20h, 31kd-2h and 80kd-20h 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.

for 2 h was not sufficient for complete conversion of the 31-kDa csA into the hydrophilic type. The differences in sensitivity to PI-PLC treatment between the 31-kDa 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 (Oxford Glycoscience Ltd.) against the cross-reacting determinant (CRD), which is a common carbohydrate epitope exposed on PI-PLC cleavage[13]. In contrast da Silva and Klein[14] reported that the 80-kDa csA released by endogenous PI-PLC reacted with anti-CRD antibody. It is difficult to draw a conclusion that the inconsistency between our and their results might be due to different kinds of PI-PLC. It is conceivable that a GPI-anchor structure of csA is different from GPI-anchor structures with CRD.

The csA retains a GPI-anchor with ceramide as well as glycoproteins in *Saccharomyces cerevisiae* and *Trypanosoma cruzi* whose GPI-anchor structures are studied well[15),16]. The validity of PI-PLC in the GPI-anchor cleavage of the 80-kDa csA or the 31-kDa csA was determined by a shift to a lower molecular weight of each csA molecule on SDS-PAGE. In this method, however, it is possible that protease contaminants in PI-PLC enzyme sources may have been responsible for the molecular weight shift during incubation. To investigate this possibility, the 80-kDa csA or 31-kDa csA was incubated with PI-PLC at 40 units ml^{-1} (a final conc.) at 37℃ for 4 h in the presence of each protease inhibitor: leupeptin (serine protease inhibitor, a final conc. of 4.8 $\mu g\ ml^{-1}$); phosphoramidon (metallo endopeptidase inhibitor, a final conc. of 194 $\mu g\ ml^{-1}$); pepstatin (aspartate protease inhibitor, a final conc. of 4.8 $\mu g\ ml^{-1}$) (Figure 2). In addition to these protease inhibitors, antipain dihydrochloride (an

inhibitor of papain, trypsin and cathepsin A, B, a final conc. of 200 μ g ml^{-1}); bestatin (aminopeptidase inhibitor, a final conc. of 50 μ g ml^{-1}); aprotinin (serine protease inhibitor, a final conc. of 10 μ g ml^{-1}) 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 weight shifts of the 80-kDa and 31-kDa csA were due to GPI-anchor cleavage by PI-PLC, and not digestion by protease contaminants in the PI-PLC enzyme sources.

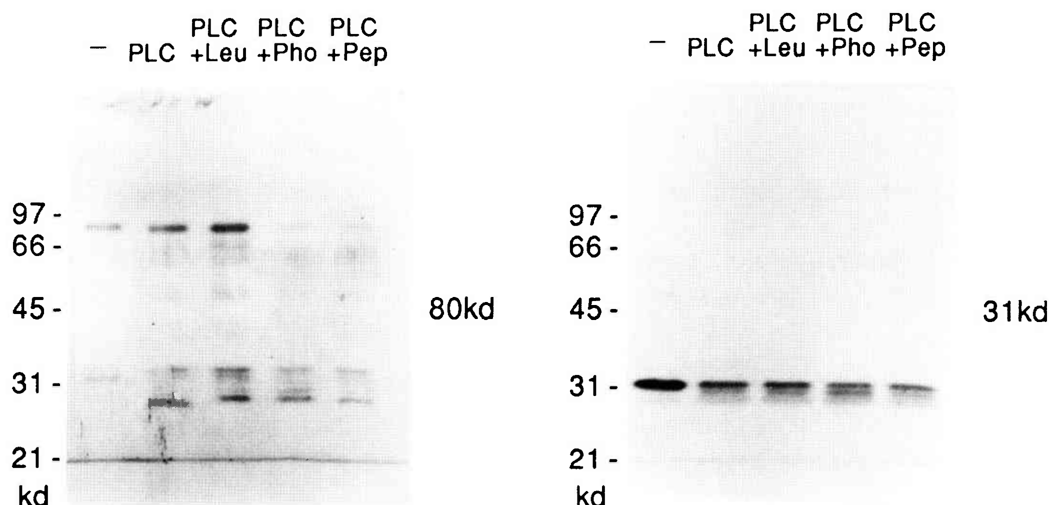

Figure 2. Effects of PI-PLC in the presence of protease inhibitors.
PI-PLC treatment of the 31-kDa csA or 80-kDa csA was carried out in the presence of each protease inhibitor. SDS-PAGE and staining were carried out as described in Figure 1. The upper panel shows the results of PI-PLC treatment of 80-kDa csA in the presence of protease inhibitors and lower panel shows those of the 31-kDa csA.—, without PI-PLC and protease inhibitors; PLC, with PI-PLC and without protease inhibitors; PLC+Leu, with PI-PLC and leupeptin (a final conc. of 4.8 μg ml^{-1}; PLC+Pho, with PI-PLC and phosphoramidon (a final conc. of 194 μg ml^{-1};PLC+Pep, with PI-PLC and pepstatin (a final conc.of 4.8 μg ml^{-1}.

Treatment of the 80-kDa csA or 31-kDa csA with 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 be cleaved with PI-PLC, showing that ceramides in GPI-anchor do not confer the resistance to PI-PLC treatment[15),16)]. Phospholipase D (PLD) treatment of the 80-kDa csA or the 31-kDa csA was carried out to investigate the effects of modification of the inositol ring with fatty acids, because PLD activity is not affected by myo-inositol acylation[17)]. Therefore, the 80-kDa csA and 31-kDa csA were incubated separately with PLD at 3 units μ l^{-1} (a final conc.) at 37℃ for 2 or 20 h. The GPI-anchors in both 80-kDa csA and 31-kDa csA were clearly susceptible to PLD treatment (Figure 3). In the both cases, a glycopeptide with molecular weight of 29-kDa were observed, reacting with only WGA. The molecular weight shift was observed irrespective of the presence of protease inhibitors in the PLD treatment as well as PI-PLC treatment in the presence of protease inhibitors (*cf.* Figure 2, data not shown). These results suggested that the 29-kDa csA was the final product of PLD treatment.

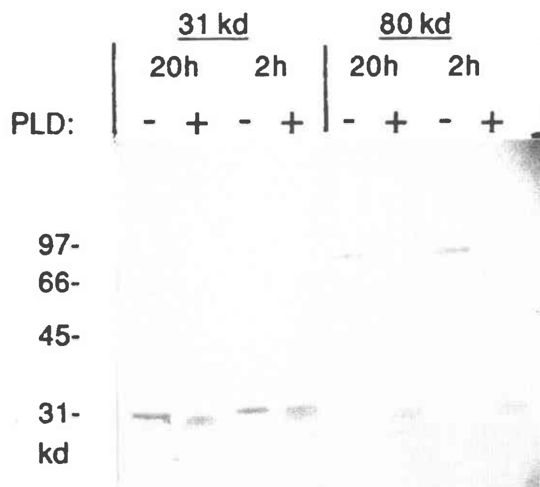

Figure 3. Treatment of the 31-kDa csA and 80-kDa csA with PLD.
The 31-kDa csA and 80-kDa csA were treated with PLD for 2 h or 20 h. The samples were separated by SDS-PAGE. The gels were blotted onto nitrocellulose filters, incubated with peroxidase-conjugated WGA. 31kDa -, 31-kDa csA incubated without PLD; 31kDa+, 31-kDa csA incubated with PLD; 80kDa -, 80-kDa csA incubated without PLD; 80kDa+, 80-kDa csA incubated with PLD.

Influence of a PLD inhibitor on cell adhesion, chemotaxis and development The sensitivity of the 80-kDa csA to PLD treatment suggested that in *Dictyostelium* the GPI-anchored proteins including the 80-kDa csA might be cleaved by an endogenous PLD *in vivo* [19),20)]. To investigate the possible function of 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 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 (Figure 4B, Table 1), although the amount of 80-kDa csA was reduced to about 50% of that in untreated cells. The inhibition of EDTA-sensitive cell contact might have been due to the function of 1,10-phenanthroline as a chelator. Furthermore, 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 (Figure 4D). On the other hand, cells treated with 100 μM 1,10-phenanthroline showed sufficient chemotaxis toward cyclic AMP. These results suggested that PLD might be functional in *Dictyostelium* development.

In future studies, we plan to investigate PLD activity during development of *Dictyostelium* and will attempt to isolate PLD knockout mutants.

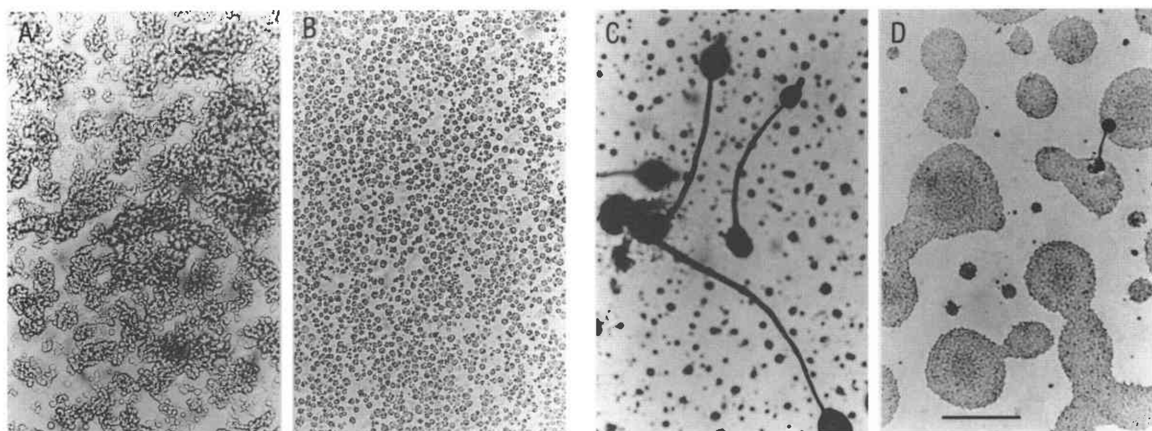

Figure 4. Photographs of cell agglutination and morphogenesis on agar plates. Assay of cell agglutination in the presence of EDTA was carried out without (A) or with 100 μM 1,10-phenanthroline (B) as described in Materials and Methods. *D. discoideum* morphogenesis was observed on agar plates without (C) or with 100 μM 1,10-phenanthroline (D). Scale bar indicates 400 μm.

Table 1 Effects of 1, 10-phenanthroline on cell agglutination (Single cells %)

		0	1	10	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 starved for 8 h, washed, and rotated at 40 rpm for 20 min at 22℃. The mean and range were derived from three separate experiments.

References

1) J. Stadler, T.W. Keenan, G. Bauer and G. Gerisch: EMBO J., **8**, 371-377 (1989)
2) M.A.J. Ferguson and A.F. Williams: Annu. Rev.Biochem., **57**, 285-320 (1988)
3) M.G. Low: Biochim. Biophys. Acta., **988**, 427-454 (1989)
4) W.L. Roberts, J.J. Myher, A. Kuksis, M.G. Low and T.L. Rosenberry: J. Biol. Chem., **263**,18766-18775 (1988)
5) D.J. Watts and J. M. Ashworth: Biochem. J.,**119**, 171-174 (1970)
6) M. Yoshida: J. Biochem.,**101**, 1233-1245 (1987)
7) M. Yoshida, S.Yokota and S. Ouchi: Exp. Cell Res., **230**, 393-398 (1997)
8) S. Tsunasawa, T. Masaki, M. Hirose, M. Soejima and F. Sakiyama: J. Biol. Chem., **264**, 3832-3839 (1989)
9) M. Yoshida, K. Takahashi, Y. Ohmori and A. Hayashi : J. Carbohydr. Chem.,**16**, 647-653 (1997)
10) H. Towbin, T. Staehelin and J. Gordon: Proc. Natl. Acad. Sci. USA., **76**, 4350-4354 (1979)
11) H. Beug and G. Gerisch: J. Cell Biol., **56**, 647-658 (1973)
12) G. Gerisch and H.U. Keller: J. Cell Sci., **52**, 1-10 (1981)
13) S.E. Zamze, M.A.J. Ferguson, R. Collins, R.A. Dwek and T.W. Rademacher: Eur. J. Biochem., **176**, 527-534 (1988)
14) A.M. daSilva and C. Klein: Cell Biol. Int. Rep., **15**, 1065-1082 (1991)
15) A. Conzelmann, A. Puoti, R.L. Lester and C. Desponds : EMBO J.,**11**, 457-466 (1992)
16) R.M. Lederkremer, C. Lima, M. I. Ramirez and O.L. Casal: Eur. J. Biochem., **192**, 337-345 (1990)
17) M.A. Deeg and M.A. Davitz : Methods Enzymol., **250**, 630-640 (1995)
18) R.K. Kamboj and C-H. Siu: Biochim. Biophys. Acta., **951**, 78-84 (1988)
19) C.N. Metz, G. Brunner, N.H. Choi-Muira, H. Nguyen, J. Gabrilove, I.W. Caras, N. ltszuler, D. B. Rifkin, E.L. Wilson, and M.A. Davitz: EMBO J., **13**, 1741-1751 (1994)
20) R. Lierheimer, B. Kunz, L. Vogt, R. Savoca, R. Brodbeck and P. Sonderegger: Eur. J. Biochem., **243**, 502-510 (1997)

粘菌細胞におけるcsAのアンカー領域の性状について

吉田元信

摘　要

粘菌細胞における接着分子csAは集合期のEDTA-resistant cell contactに関与する分子量8.0万の糖タンパク質である。接着分子80kDa csAの*Achromobacter*プロテアーゼＩ処理によりアンカー領域を保持した31kDa csAが単離された。接着分子80kDa csAと31kDa csAのホスファチジルイノシトール特異的なホスホリパーゼＣに対する感受性は31kDa csAが80kDa csAより強かった。一方、ホスホリパーゼＤ処理に対しては80kDa csAも31kDa csAもともに非常に高い感受性を示し、アンカー領域を遊離した。さらに、ホスホリパーゼD阻害剤1,10ーフェナントロリン存在下では粘菌細胞の発生は著しく阻害された。このことはホスホリパーゼＤが発生過程での調節に重要な役割を担っていることを示唆する。