Replacement of the Phospholipid-Anchor in the Contact Site A Glycoprotein of \textit{D. discoideum} by a Transmembrane Region Does Not Impede Cell Adhesion but Reduces Residence Time on the Cell Surface

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Abstract. The contact site A (csA) glycoprotein of \textit{Dictyostelium discoideum}, a cell adhesion molecule expressed in aggregating cells, is inserted into the plasma membrane by a ceramide-based phospholipid (PL) anchor. A carboxyterminal sequence of 25 amino acids of the primary csA translation product proved to contain the signal required for PL modification. CsA is known to be responsible for rapid, EDTA-resistant cohesion of cells in agitated suspensions. To investigate the role of the PL modification of this protein, the anchor was replaced by the transmembrane region and short cytoplasmic tail of another plasma membrane protein of \textit{D. discoideum}. In cells transformed with appropriate vectors, PL-anchored or transmembrane csA was expressed under the control of an actin promoter during growth and development. The transmembrane form enabled the cells to agglutinate in the presence of shear forces, similar to the PL-anchored wild-type form. However, the transmembrane form was much more rapidly internalized and degraded. In comparison to other cell-surface glycoproteins of \textit{D. discoideum} the internalization rate of the PL-anchored csA was extremely slow, most likely because of its exclusion from the clathrin-mediated pathway of pinocytosis. Thus, our results indicate that the phospholipid modification is not essential for the csA-mediated fast type of cell adhesion but guarantees long persistence of the protein on the cell surface.
tion is whether this high mobility is only guaranteed by the PL anchor.

In the present paper a carboxyterminal sequence of 25 amino acids, that is sufficient to direct lipid attachment of the csA protein, has been exchanged in a fusion protein by a transmembrane region. We demonstrate that the transmembrane version of the csA protein is capable of mediating EDTA-stable cell adhesion in an agitated suspension, indicating that the phospholipid anchor is not essential for fast adhesion. However, rapid turnover of the transmembrane form indicates that the presence of the anchor guarantees long persistence of the csA glycoprotein on the cell surface.

**Materials and Methods**

**Vector Constructions**

CsA sequences were derived from plasmid p312-6, kindly provided by B. Leiting, carrying the full length 1.8 kb csA cDNA (Noegel et al., 1986). P29F8 sequences were taken from plasmid p36, carrying the full length 1.9 kb P29F8 cDNA (Müller-Taubenberger, 1989). For the construction of D. discoideum expressing the fusion proteins PC and CP a unique PstI site was introduced into the coding unit of csA by site-directed mutagenesis with the pMa/c5-8 plasmid system (Stanssens et al., 1989). The PstI site was introduced by the exchange of one nucleotide at position 1466 of the csA cDNA, which converted Gla to Ala. The csA sequence 3' of this PstI site was used to replace the 3'-PstI/HindIII fragment in the P29F8 cDNA, resulting in a coding unit for the fusion protein PC. The csA sequence upstream of the introduced PstI site was fused to frame the 3'-PstI/HindIII fragment of the P29F8 cDNA, resulting in the coding unit for the fusion protein CP. The Ala codon produced by mutation of the csA sequence replaced the Ala codon at the unique PstI site in the P29F8 sequence, so that PC contained the codons for 25 amino acids of csA, whereas in CP 26 amino acids of csA were replaced. The recombinant DNAs were verified by sequencing the fusion regions. For constitutive expression of the encoded proteins these cDNAs were cloned into one of the following D. discoideum expression vectors in which expression is controlled by the actin 15 promoter (Knecht et al., 1986). (a) For the fusion protein PC the corresponding coding unit was cloned into pDEX RH, kindly provided by J. Faix, Max-Planck-Institut für Biochemie, Martinsried. This vector was derived from pDEX H (Faix et al., 1992) by introducing an EcoRI site into the cDNA expression cassette. (b) For the fusion protein CP the cDNA fragment downstream of the Neoc site in the csA expression vector pDCEV4 (Faix et al., 1990) was replaced by the 3'-Neoc fragment of the CP coding unit. (c) For the wild-type protein P29F8 the complete cDNA of P29F8 was cloned into pDEX RH. (d) For the csA protein the vector pDCEV4 (Faix et al., 1990) was used.

**Transformation of D. discoideum Strains**

The strains employed were AX2 clone 214 and HG1287. This strain is a parasexual recombinant obtained by E. Wallraf from HG693, a csA-defective mutant of AX2 (Faix et al., 1992). AX2 and HG1287 cells were transformed by the Ca-phosphate method according to Nellen et al. (1984) with pDCEV4 and pDEX/CP. The AX2 transformants were designated as AT-CI and AT-CP1, respectively; the HG1287 transformants accordingly as HT-CI and HT-CP7 and 8. HG1287 cells were also transformed with pDEX RH/P29F8 and with pDEX RH/PC, resulting in transformants HT-P3 and HT-PC, respectively. Transformants were selected for G418 resistance of the introduced 3'-PstI/HindIII fragment of the P29F8 cDNA, resulting in the coding unit for the fusion protein CP. The Ala codon produced by mutation of the csA sequence replaced the Ala codon at the unique PstI site in the P29F8 sequence, so that PC contained the codons for 25 amino acids of csA, whereas in CP 26 amino acids of csA were replaced. The recombinant DNAs were verified by sequencing the fusion regions. For constitutive expression of the encoded proteins these cDNAs were cloned into one of the following D. discoideum expression vectors in which expression is controlled by the actin 15 promoter (Knecht et al., 1986). (a) For the fusion protein PC the corresponding coding unit was cloned into pDEX RH, kindly provided by J. Faix, Max-Planck-Institut für Biochemie, Martinsried. This vector was derived from pDEX H (Faix et al., 1992) by introducing an EcoRI site into the cDNA expression cassette. (b) For the fusion protein CP the cDNA fragment downstream of the Neoc site in the csA expression vector pDCEV4 (Faix et al., 1990) was replaced by the 3'-Neoc fragment of the CP coding unit. (c) For the wild-type protein P29F8 the complete cDNA of P29F8 was cloned into pDEX RH. (d) For the csA protein the vector pDCEV4 (Faix et al., 1990) was used.

**Culture Conditions for the Analysis of Transformants and Measurement of Cell Adhesion**

Cells were cultivated at 23°C axenically in liquid nutrient medium containing 1.8% maltose (Watts and Ashworth, 1970) on a gyrorathy shaker at 150 revs/min and harvested from this medium during exponential growth at a density of not more than 5 × 10⁶ cells/ml. For starvation and initiation of development, the cells were washed and adjusted to 10⁷/ml in PB (17 mM Soerensen phosphate buffer, pH 6.0), and shaking was continued.

Cell–cell adhesion was determined in an agglutinometer as described by Beeg and Gerisch (1972) and Bozzaro et al. (1987). Before the measurement, cells were washed and resuspended at a density of 10⁷/ml in PB, pH 6.0, with or without 10 mM EDTA.

**Metabolic Labeling**

For labeling with [³H]palmitic acid (Stadler et al., 1984) the concentration of growth-phase cells was adjusted to 10⁷/ml by dilution into fresh nutrient medium. 1 mCi [³H]palmitic acid (New England Nuclear, Boston, MA; NET 043, 30 Ci/mmol) was added to 5 ml aliquots of cells and incubated for 16 h. Then the cells were washed twice with PB, pH 6.0, collected by centrifugation, and frozen at –80°C.

For pulse-chase labeling of developing cells with [³S]methionine (Amersham Corp., Arlington Heights, IL, 10 mCi/ml) growth-phase cells were washed twice and resuspended in PB, pH 6.0, at a density of 10⁹/ml. 0.5 mCi/ml of [³S]methionine was added and the cells were incubated for 30 min. For the chase, the cells were washed twice and resuspended at 10⁷/ml in PB, pH 6.0, containing 2 mM of unlabelled methionine. Aliquots of cells were collected by centrifugation after various times of chase and frozen at –80°C.

**Cell-Surface Biotinylation and Assay for Internalization**

For cell-surface biotinylation, growth-phase cells were washed twice and resuspended in ice-cold PB, pH 8.0, at a density of 10⁷/ml. Sulfosuccinimidyl (2-biotinamido)ethyl-1,3-dithiopropionate (NH4SS-biotin) was used as a reagent of the sulfosuccinimidyl analog of sulfo-NHS-biotin, Pierce, Rockford, IL) was added to a final concentration of 0.25 mg/ml, and the cells were shaken for 30 min at 0°C and 150 revs/min. After labeling, the cells were washed twice and resuspended at a density of 10⁷/ml in ice-cold PB, pH 7.2, containing 40 mM NH₄Cl. NH₄Cl was added to the buffer to inhibit lysosomal degradation of internalized proteins (Seglen and Keith, 1978). Aliquots of the suspensions were kept on ice or incubated in the same buffer at 23°C and 150 revs/min to allow endocytosis to occur. After various times, endocytosis was inhibited by chilling the suspensions in ice water and the cell-surface label was stripped by reduction with glutathione as follows: cells were collected by centrifugation, resuspended at a density of 10⁷/ml in ice-cold glutathion solution (30 mM glutathione, 75 mM NaCl, 75 mM NaOH, 1% BSA) and incubated for 30 min at 150 revs/min in ice water. After reduction the cells were washed twice in ice-cold PB, pH 7.2, containing 40 mM NH₄Cl, collected by centrifugation and frozen at –80°C.

**Immunoprecipitation of Proteins from Cell Lysates**

CsA, and CP were precipitated with mAb 71 which recognizes the protein moiety of the native csA glycoprotein (Bertholdt et al., 1985). P29F8 and PC were precipitated with mAb 210. All procedures were carried out at 4°C. Cells were freeze-thawed at concentrations varying from 6 × 10⁶ to 10⁷/ml and the lysates were subsequently incubated for 30 min with 1% (vol/vol) octyloligoxyethylene (Buchem, CH-4416 Bubendorf, Switzerland) in 10 mM Hepes-NaOH buffer, pH 7.5, containing 1 mM DTT (lysis buffer). Extracts were cleared by centrifugation, supplemented with 20 mM NaCl and incubated for 2 h with 150 μg/ml of purified IgG. For precipitation, protein A-Sepharose CL-4B beads (Pharmacia LKB Biotechnology, Piscataway, NJ) were added in excess (300 μl swollen beads + 1.2 ml DTT-Hepes-NaCl per 150 μg of IgG). The samples were agitated to keep the beads suspended during the incubation with antibody. After 1 h the beads were collected by centrifugation at 600 g for 30 s, washed four times with sevenfold the volume of lysis buffer supplemented with 20 mM NaCl, and boiled for 3 min in SDS-sample buffer (Laemmli, 1970). Proteins from NHS-SS-biotin labeled cell lysates were immunoprecipitated without the addition of DTT to the lysis buffer, and the SDS-sample buffer contained no β-mercaptoethanol.

After immunoprecipitation of proteins from [³H]palmitic-acid labeled cell lysates beads were boiled in SDS sample buffer without glycerol and bromophenol blue and the supernatants were acidified to 0.1 N of HCl (Stadler et al., 1989). Noncovalently bound lipids were removed by chioroform/methanol extraction of the proteins from the acidified solution (Wessel and Filip, 1984).
For SDS-PAGE (Laemmli, 1970) either total cell homogenates or immunoprecipitates were used. If not stated otherwise, equivalents of 10^6 cells were applied per lane in 7.5% or 10% gels and proteins were transferred to Schleicher and Schuell (Keene, NH) HA 85 nitrocellulose (Vaessen et al., 1981). For fluorography of the [3H] or the [35S] label, the filters were dipped into 20% (wt/vol) of 2,5-diphenylloxazole in toluene, dried, and exposed at -70°C on Kodak XAR-5 film. 2,5-Diphenylloxazole was removed by washing the filters with toluene for subsequent immunolabeling. Antibodies applied to immunoblots were mAb 294 which recognizes the protein A chain of denatured csA (Bertholdt et al., 1985), mAb 353 against N-linked oligosaccharides of csA (Faix et al., 1990), mAb 210 against O-linked oligosaccharides of both csA and P29F8 (Bertholdt et al., 1985), mAb 200 against severin as a control (André et al., 1989), and polyclonal pAb P against the carboxyterminal end of P29F8. The pAb P serum was obtained by immunizing a rabbit with a synthetic peptide, representing the sequence of the 17-amino acids of the carboxyterminus of the protein bound via a K-G-G spacer to a palmitoyl residue, together with Freund’s adjuvant (Müller-Taubenberger, 1989). Blotted proteins were directly or indirectly labeled with [35S]-labeled antibodies. For quantitative comparisons the glycoprotein bands were scanned on fluorograms or autoradiograms in two dimensions on an Elscript-400 electrophoresis scanner (Hirschmann Gerätäub, 82008 Unterhaching, Germany) using a round aperture of 0.5-mm diam.

Subcellular Fractionation and Enzyme Assays

3 x 10^6 cells were starved for 4 h in 30 ml PB, pH 7.2, in the presence of 40 mM NH₄Cl to inhibit degradation of proteins by lysosomal enzymes. Subsequently, the cells were cooled to 0°C and lysed in fractionation buffer: PB, pH 7.2, with 40 mM NH₄Cl, 0.25 M sucrose and protease inhibitors (10 mM benzamidine, 28 μg per ml of aprotinin, and a 100-fold dilution of a stock solution containing 50 μg bestatin, 100 μg pepstatin, 100 μg antipain, 100 μg leupeptin in 1 ml of methanol). Lysis was obtained by three passages of the cells through Nucleopore filters with pore diameters of 5 μm. Unbroken cells and nuclei were removed by centrifugation at 3,000 g for 5 min. Membranes and intracellular vesicles were collected by centrifugation of the supernatant at 15,000 g for 30 min. The 15,000 g pellet was resuspended in 1 ml fractionation buffer and loaded on top of 39 ml of the buffer containing 22% Percoll (Pharmacia LKB Biotechnology). After centrifugation at 23,000 rpm for 30 min in a Kontron VT150 rotor, the gradient was fractionated from the bottom of the tube into 19 equal fractions. The fractions were assayed for enzyme activities or examined by SDS-PAGE and immunoblotting.

Triton X-100 was added to the fractions to a final concentration of 0.5%. Acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) were assayed according to Loomis and Kuspa (1984) and Loomis (1969). Percoll did not affect the enzyme activities. After removal of precipitated Percoll by centrifugation, the absorption of the supernatant was measured at 410 nm.

Results

Construction of a csA Fusion Protein That Is Endowed with a Transmembrane Sequence

To obtain a protein in which the phospholipid anchor of the csA protein is replaced by a transmembrane polypeptide sequence, fusions were designed between cDNA fragments that code for portions of the csA sequence and fragments of a D. discoideum cDNA clone that, according to its isolation number, has been referred to as P29F8 (Fig. 1). The P29F8 cDNA codes for a polypeptide of 58 kD with three domains: a large extracellular NH₂-terminal region, one transmembrane stretch, and a small cytoplasmic domain. The protein is N- and O-glycosylated, giving rise to a product with an apparent molecular mass of 100 kD in 10% SDS gels. Under the same conditions, csA forms a band corresponding to a glycoprotein of 80 kD. Although the function of the protein encoded by this clone is unknown, P29F8 offers itself for fusion with csA for two reasons. The transcript is similarly regulated during development as the csA transcript; both messages are absent from growth-phase cells and are maximally expressed during the aggregation phase (for csA see Murray et al., 1981; Noegel et al., 1986; for P29F8 Müller-Taubenberger, 1989). Elimination of P29F8 by gene disruption or overexpression of this protein in growth-phase cells has revealed no activity of the protein in cell adhesion (Müller-Taubenberger, 1989; Barth, 1992). Thus, P29F8 sequences will have no impact on the measurement of cell adhesion in transformants.

Our goal was to construct a csA-fusion protein with the phospholipid anchor replaced by a transmembrane sequence but missing only a minimal portion of the csA sequence. Ideally, this portion should comprise not more than the COOH-terminal end of the csA protein which is required for attachment of the anchor. As shown by construction of the fusion protein PC (Fig. 1), the very last 25 amino acids of the csA protein are sufficient for phospholipid anchorage. This result implies that a cleavage site for replacement of the COOH terminus by an anchor resides within this short COOH-terminal sequence of the primary translation product.

For use in cell adhesion studies the fusion protein CP was constructed (Fig. 1). This protein contained the entire csA sequence except of 26 amino acids of the COOH terminus (26 instead of 25 residues were removed for technical reasons as explained in Materials and Methods). These amino acids were replaced by 75 COOH-terminal residues of P29F8 which encompass the putative transmembrane and cytoplasmic domains of this protein.

No incorporation of [3H] palmitic acid into CP was detected, which indicates the lack of a phospholipid anchor (Fig. 2). In accord with these results, CP was recognized by rabbit antibodies pAb P that had been raised against a synthetic peptide corresponding to the COOH-terminal end of P29F8 (Fig. 3). This label confirms that no replacement of the COOH terminus of the fusion protein by a phospholipid anchor had occurred and provides evidence that CP is inserted into the membrane by the transmembrane sequence of P29F8.

The normal csA protein is modified by N- and O-linked oligosaccharide residues, referred to as carbohydrates 1 and 2. To prove that CP expressed in growth-phase cells is endowed with both types of carbohydrate residues, proteins from the CP-expressing cells were labeled with mAbs 353 and 210 (Fig. 3). mAb 353 is directed against the fucosylated, N-linked type 1 carbohydrate chains (Faix et al., 1990), mAb 210 against type 2 carbohydrate residues which are posttranslationally added during passage of the csA protein through the Golgi apparatus (Hohmann et al., 1987a,b). Both antibodies recognized CP. The band formed by CP in 7.5% SDS gels indicated a glycoprotein with an apparent molecular mass of 87 kD (Fig. 3).

Comparison of EDTA-Stable Cell Adhesion Mediated by Phospholipid Anchored csA and by the Transmembrane Fusion Protein CP

In wild-type cells, csA as well as P29F8 are synthesized only during development, so that fusion proteins can be examined in growth-phase cells of transformants without any interference by the endogenous protein. Wild-type AX2 cells were transformed in parallel with appropriate vectors so that they expressed either csA or CP during their growth-phase. For
Comparing cell adhesion, a pair of transformants was chosen which expressed csA or CP in about equal amounts (Fig. 4A). In contrast to untransformed growth-phase cells which do not adhere to each other in the presence of EDTA, strong EDTA-stable adhesiveness was found in both transformants (Fig. 4B). In fact, we found no difference in the strength of adhesiveness by an agglutinometer assay (Fig. 4C). This result indicates that EDTA-stable cell adhesion, as measured in an agitated suspension, is not significantly reduced when the adhesion protein is inserted into the plasma membrane by a membrane-spanning polypeptide sequence.

In an attempt to extend these studies to developing cells, which in the wild-type produce their own csA, we have transformed cells of the csA negative mutant HGI287 with the same vectors as used for the assay of cell adhesion in wild-type growth-phase cells. Surprisingly, if transformants expressing csA or CP were compared at 9 h of development, EDTA-stable cell adhesiveness was found to be weaker in the latter (Fig. 5A). This observation found its explanation when, in the same cultures, the amounts of proteins were determined. Immunoblotting showed a decrease in CP during the first 9 h of development which was not paralleled by a decrease in csA (Fig. 5B).

To examine whether the decrease in the amount of CP during development is due to its rapid degradation, csA, CP and P29F8 were pulse labeled with [35S]methionine at the beginning of development. At intervals, aliquots of the labeled cells were lysed, the three proteins were immunopre-
Figure 2. Immunoprecipitation of csA and of the fusion protein CP from [3H] palmitic acid labeled cells, showing lack of phospholipid modification in CP. Growth-phase cells of transformants HT-Cl expressing csA, and of transformants AT-CP1 and HT-CP8 expressing CP were incubated with [3H] palmitic acid. (Construction of these transformants is described in Materials and Methods.) Proteins were solubilized by detergent and immunoprecipitated with csA-specific mAb 71. After extraction of noncovalently bound fatty acids, the proteins were subjected to SDS-PAGE, transferred to a nitrocellulose filter, and fluorographed (A). Subsequently, the same filter was labeled with csA-specific 125I-mAb 294 and autoradiographed (B). Equivalents of $3 \times 10^6$ HT-C1 cells, of $10^7$ AT-CP1 cells or of $8 \times 10^6$ HT-CP8 cells were applied per lane. Positions of molecular mass markers are indicated on the left.

Figure 3. Presence of the P29F8 COOH-terminal sequence and of carbohydrate modifications in the fusion protein CP. Total proteins from untransformed AX2 cells or from cells of the transformant AT-CP1 were separated by SDS-PAGE, blotted, and labeled with the antibodies indicated on bottom. The AX2 cells were harvested at 6 h of starvation (6) when csA and P29F8 were fully expressed, the AT-CP1 cells were harvested at the growth phase (0) when only the vector-encoded fusion protein was present. Equivalents of $10^6$ cells were applied per lane, csA and CP were recognized by the csA specific antibody mAb 294. CP but not csA was labeled by the anti-peptide antibody pAb P which recognizes the COOH-terminal sequence of P29F8 (Fig. 1B). mAb 353 and mAb 210 label N- and O-linked carbohydrate chains, respectively. The 100-kD band recognized by pAb P and mAb 210 represents the endogenous glycoprotein P29F8. The band of a 29-kD glycopeptidase in the transformant is obviously a COOH-terminal degradation product of CP which carries O-linked but no N-linked carbohydrate residues. This fragment accumulates during turnover of the protein because the carbohydrate-decorated portion of the polypeptide chain is protected against proteolysis (Hohmann et al., 1987b).

The extent of release from the cells was examined by probing for csA and CP in the extracellular medium. Blots were labeled with mAbs 294, 353, and 210, for both proteins, and with pAb P for the COOH-terminus of CP (see Fig. 3). All the antibodies revealed the same result: compared to the amounts of the two proteins that were associated with the cells, very little was detected in the extracellular medium in the form of intact proteins or large fragments. The amount of CP released was in the same range as the quantity of csA normally shed-off from the cells (data not shown).

To test whether CP is more rapidly internalized than csA, proteins located at the cell surface were pulse-labeled with NHS-SS-biotin, a reducible derivative of biotin which does not pass the plasma membrane (Lisanti et al., 1990b). The labeled cells were incubated with 40 mM NH4Cl to inhibit proteolytic degradation of internalized proteins by elevating the lysosomal pH (Seglen and Reith, 1976). At intervals of 0–60 min, aliquots of cells were treated with glutathione to remove cell-surface exposed biotin by reduc-

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Figure 4. Comparison of cell adhesion at the growth-phase stage of the wild-type strain AX2 and of transformed cells expressing csA (AT-C1) or the fusion protein CP (AT-CP1). (A) Absence of csA from growth-phase AX2 cells is shown, and the amounts of csA and CP per cell equivalent are compared in the transformants by SDS-PAGE and immunoblotting. Total proteins from 10^6 cells were harvested immediately after removal of nutrient medium (0) and at 2 h of starvation (2); in the period between, the agglutinometer assay was performed. (B) Photographs of cells derived from the same cultures as shown in A. These cells were assayed in an agglutinometer immediately after removal of nutrient medium, and photographs were taken after 1 h of exposure to standard shear forces in the presence of 10 mM EDTA. The bar indicates 200 μm. (C) Records of light scattering monitored in the agglutinometer under the same conditions as in B. Ordinate: Light scattering values E in arbitrary units; low values indicate agglutination of the cells. Abscissa: time in min after transfer of the cells to the agglutinometer. The cells were incubated in the agglutinometer either without EDTA (open symbols) or with 10 mM EDTA (closed symbols).

Figure 5. Reduced EDTA-stable adhesion during development of cells expressing CP (A), paralleled by a decrease in the amount of this protein (B). By transformation of the csA-negative mutant HG1287, transformants were obtained that express constitutively either normal csA (HT-C1) or the fusion protein CP (HT-CP8). (A) Cells were harvested immediately after removal of nutrient medium (0) or at 9 h of starvation (9) and incubated in the agglutinometer with 10 mM EDTA. Photographs were taken after 1 h in the agglutinometer. The bar indicates 100 μm. (B) Total proteins of cells from the same cultures as in A were subjected to SDS-PAGE and immunoblotting. Cells were harvested at 0, 3, 6, and 9 h of starvation, and equivalents of 10^6 cells were applied per lane. The blot was incubated with anti-csA mAb 294 and subsequently with severin-specific mAb 200. The constitutively expressed actin-binding protein severin served as a reference.

Scanning of the blots shown in Fig. 7 (upper panels) showed that within 1 h only 6 per cent of the biotin-labeled csA, but 56 per cent of the biotin-labeled CP was internalized. The total amounts of csA and CP in the cells were about the same. These amounts remained almost constant during the experiments, as indicated by labeling the proteins with mAb 294 (Fig. 7, lower panels). These results demonstrate that the fusion protein CP is more rapidly internalized than the phospholipid-anchored csA.

To examine whether internalized CP is transported to dense endosomes and secondary lysosomes, the distributions of csA and CP were compared after 4 h of starvation in the presence of 40 mM NH4Cl in order to inhibit degradation of the glycoproteins (Bush and Cardelli, 1989; Cardelli...
Figure 6. Pulse-chase experiment to determine turnover of the wild-type proteins csA and P29F8 and of the fusion protein CP in starved cells of HG1287-derived transformants. Growth-phase cells of the csA expressing transformant HT-C1, the P29F8 expressing transformant HT-P3, and the two CP expressing transformants HT-CP7 and HT-CP8 were labeled with $[^{35}S]$methionine immediately after removal of nutrient medium. After 30 min of incubation cells were chased with excess unlabeled methionine. From aliquots taken at the times indicated, proteins of detergent-solubilized cell lysates were precipitated with mAb 71 for csA and CP, or with mAb 210 for P29F8. The precipitates were subjected to SDS-PAGE and blotting. After fluorography for $[^{35}S]$, the films were scanned and label in the glycoprotein bands given as relative absorption (A), taking the density at 1 h of chase as 100%. In case of HT-C1, HT-P3, and HT-CP8 each value represents the mean of two pulse/chase experiments, and vertical bars denote the difference between the mean and the actual values. For HT-CP7 the data of one pulse/chase experiment are shown.

Figure 7. Evidence for a low rate of endocytosis of PL-anchored csA in comparison to the transmembrane protein CP. Growth-phase cells of the csA-expressing transformant HT-C1 and of the CP-expressing HT-CP8 were surface-labeled at 0°C with NHS-SS-biotin immediately after removal of nutrient medium, and subsequently incubated at 23°C to allow endocytosis to occur. During this period the cells were treated with 40 mM NH$_4$Cl to inhibit degradation of endocytosed proteins by lysosomal enzymes. After 0–60 min as indicated on top, internalization was stopped by cooling to 0°C. Cell surface label was removed by reduction with glutathione, except for lane C which served as an unreduced control. csA and CP were precipitated from detergent-solubilized cell lysates with mAb 71. The precipitated proteins from $5 \times 10^6$ cells were subjected to SDS-PAGE and duplicate blots were labeled either with $^{125}$I-streptavidin (upper panels) or with $^{125}$I-mAb 294 (lower panels). Protection of the biotin-label indicates internalization of the respective protein. The mAb 294 label shows that total amounts of the two proteins did not significantly change during the experiment.
that the sequence of the 25 carboxyterminal amino acids of the primary translation product is responsible for modification of the csA glycoprotein by a PL anchor. Despite the differences in structure between the ceramide-based anchor of the Dictyostelium protein (Stadler et al., 1989) and the GPI anchors of protozoan and mammalian proteins, the carboxyterminal sequences of three phospholipid anchored Dictyostelium proteins, including csA, fit into a common scheme (Fig. 9). Serine or glycine at the predicted cleavage (α) site to which the anchor is attached, and alanine or serine at the ω + 2 site agree with the requirements for GPI-modification (Moran et al., 1991; Gerber et al., 1992; Kodukula et al., 1993; Micanovic et al., 1990).

A variety of other cell-adhesion proteins are expressed on cell surfaces in a GPI-anchored form (Gennarini et al., 1989; Hortsch and Goodman, 1990; Ranscht and Dours-Zimmermann, 1991) or in both a transmembrane and a GPI-anchored version (He et al., 1986; Dustin et al., 1987; Hortsch and Goodman, 1991), suggesting that GPI anchors are relevant to the function of these proteins in mediating cell interactions. One possibility is that a PL anchor leads to clustering of the protein by hydrophobic interactions in the lipid phase of the plasma membrane, and thus to local increases in the strength of cell adhesion. Another possibility is that the mobility of a protein in the membrane is critical for its function in cell adhesion.

High lateral mobility has been established for some GPI-anchored proteins (Woda and Gilman, 1983; Ishihara et al., 1987; Noda et al., 1987). Differences in the strength of adhesion have been found for the GPI-anchored and the transmembrane isoforms of the cell adhesion molecule LFA-3 (Chan et al., 1991; Tözeren et al., 1992). Adhesion of cells to a planar bilayer containing the GPI isoform has been stronger than to a bilayer containing the transmembrane isoform. Lowering of the difference with higher LFA-3 density and also with prolonged time of contact has suggested an influence of the mobility of the molecule on the strength of adhesion (Chan et al., 1991).

To examine whether the PL-anchor has an impact on the csA-mediated “fast type” of cell adhesion as it is observed in agitated suspensions of Dictyostelium cells (Stadler et al., 1989; Harloff et al., 1989), the anchor of csA has been replaced by the carboxyterminal tail of another D. discoideum protein, which comprises a transmembrane region. The strong agglutination of the transformed cells has provided evidence that the chimeric transmembrane protein is capable of mediating EDTA-stable cell adhesion, similar to the normal PL-anchored csA molecule. No substantial difference in the adhesion capacity of cells containing equal amounts of either the PL-anchored or the transmembrane form of csA has been measured under the influence of shear force. The vectors used for expression of the transmembrane and PL-anchored form of csA in the growth-phase cells of D. discoideum tend to integrate in multiple copies into the genome. To simulate conditions in wild-type cells as closely as possible, we have chosen transformants which express less than threefold the amount of csA that wild-type AX2 expresses during the aggregation stage. Using these cells we cannot exclude an effect of the anchor in case that csA is present at lower densities on the cell surface. Nevertheless, the finding showing that PL-anchorage is not necessary for rapid adhesion has prompted us to search for other functions of the PL-anchor.

Discussion

Cell Adhesion Mediated by a Transmembrane Derivative of the csA Protein

The fusion protein used in this study was based on the finding that the sequence of the 25 carboxyterminal amino acids of the csA protein (Stadler et al., 1989) and the finding showing that PL-anchorage is not necessary for rapid adhesion has prompted us to search for other functions of the PL-anchor.

phatase activity in high-density fractions (Fig. 8, A and C), was probably caused by contamination of these fractions with cytoskeleton-associated remnants of the plasma membrane.

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Cell Adhesion Mediated by a Transmembrane Derivative of the csA Protein

The fusion protein used in this study was based on the finding that the sequence of the 25 carboxyterminal amino acids of the csA protein (Stadler et al., 1989) and the finding showing that PL-anchorage is not necessary for rapid adhesion has prompted us to search for other functions of the PL-anchor.

Figure 8. Percoll gradient fractionation of extracts prepared from ammonium chloride-treated cells. Cells of HT-Cl and HT-CP8 were starved in the presence of 40 mM NH4Cl to inhibit lysosomal pro tease. After 4 h of starvation cells were lysed and the postnuclear 15,000 g pellets fractionated on a Percoll gradient as described in Materials and Methods. Fractions were collected from the bottom (1, highest density) to the top of the tubes (19, lowest density). (A) To determine the distribution of csA and CP, aliquots of the fractions were subjected to SDS-PAGE and immunoblotting with mAG 294. (B) The same fractions were assayed for acid-phosphatase activity as a marker for lysosomes, and (C) for alkaline phosphatase activity as a plasma-membrane marker.
Phospholipid Anchorage Prevents Rapid Internalization and Subsequent Degradation of the Protein

The PL-anchored csA protein has a half-life of more than 5 h as determined by pulse-chase labeling (Fig. 6). This unusually long lifetime is based on a slow rate of internalization: only 6 per cent of the protein labeled on the cell surface is endocytosed during the first hour of starvation (Fig. 7). These data are in accord with results of Hohmann et al. (1987b) who observed a 20 per cent reduction in the amount of csA within 4 h of cycloheximide treatment of starved D. discoideum cells. In contrast, the half-life of the transmembrane version of the csA protein has been less than 2 h, and about 50 per cent of the protein becomes internalized during the first hour of starvation. Thus the difference in stability of the PL-anchored and transmembrane forms of the csA-protein is clearly due to their different susceptibilities to the endocytotic pathway. The question is whether exclusion of the PL-anchored csA protein from endocytic vesicles is responsible for this difference, or accumulation of the transmembrane protein in clathrin-coated vesicles. The latter is unlikely since the cytoplasmic tail of the fusion protein has in its sequence of 34 amino acids none of the known endocytosis signals (Kistakos et al., 1990; Trowbridge, 1991; Vaux, 1992). Furthermore, even higher rates of internalization than found for the transmembrane form of the csA protein have been measured for surface glycoproteins and total plasma membrane of D. discoideum cells (Thilo and Vogel, 1980; de Chastellier et al., 1983). These rates correspond to internalization of the total surface area once every 20–45 min. Almost all of the pinocytic activity in these cells is due to clathrin-coated vesicles (O'Halloran and Anderson, 1992). In the light of these data the persistence time of the PL-anchored csA protein on the cell surface is exceptionally long, which suggests that this protein is excluded from the coated pits.

Comparable results showing slow internalization rates have previously been obtained for the GPI-anchored decay accelerating factor in HeLa and MDCK cells (Lisanti et al., 1990b) as well as for T-cell activating protein and the Thy-1 antigen in lymphocytes (Bamezai et al., 1989; Lemansky et al., 1990). Even GPI-anchored proteins that play a key role in the endocytotic process which mediates uptake of small molecules by plasmalemmal invaginations called calveolae (Rothberg et al., 1990; for review see Hooper, 1992), are excluded from endocytosis by clathrin-coated vesicles (Bretscher et al., 1980; Keller et al., 1992).

Switching between a PL-anchored and a transmembrane isoform may provide a sort of regulation of cell adhesion. Expression of the different N-CAM isoforms, for example, is tightly regulated during embryogenesis (Hemperly et al., 1986; Covault et al., 1986; Gennarini et al., 1986). The PL-anchor guarantees a long half-life of the protein on the cell surface (Lemansky et al., 1990). By endocytosis of a transmembrane isoform, cell adhesion can be rapidly down-regulated (Bailey et al., 1992). In D. discoideum, which lives in the soil, long persistence of the csA protein has the obvious advantage of keeping cells in an adhesive state when cells are too disperse to complete aggregation within a few hours.

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