Molecular Cloning and Characterization of DdCAD-1, a Ca\(^{2+}\)-dependent Cell-Cell Adhesion Molecule, in Dictyostelium discoideum*

Estella F. S. Wong†, Simuran K. Brar§, Hiromi Sesaki, Chunzhong Yang, and Chi-Hung Siu††

From the Banting and Best Department of Medical Research and Department of Biochemistry University of Toronto, Toronto, Ontario M5G 1L6, Canada

Dictyostelium discoideum expresses EDTA-sensitive cell-cell adhesion sites soon after the initiation of development, and a Ca\(^{2+}\)-binding protein of M\(_r\) 24,000 (designated DdCAD-1) has been implicated in this type of adhesiveness. We have previously purified DdCAD-1 to homogeneity and characterized its cell binding activity (Brar, S. K., and Siu, C.-H. (1993) J. Biol. Chem. 268, 24902-24909). In this report, we describe the cloning of DdCAD-1 cDNAs. DNA sequencing revealed a single open reading frame coding for a polypeptide containing 213 amino acids. The identity of the cDNA was confirmed by amino acid sequences of two cyanogen bromide peptides. The deduced amino acid sequence of DdCAD-1 exhibits a relatively high degree of sequence similarity with members of the cadherin family and protein S of Myxococcus xanthus. Unlike the other cadherins, the carboxyl-terminal region of DdCAD-1 contains a Ca\(^{2+}\)-binding motif. Although analyses of the sequence suggest that the polypeptide lacks a signal peptide sequence and a transmembrane domain, immunofluorescence microscopy indicates a localization of DdCAD-1 with the ecto-surface of the plasma membrane. To investigate the structure/function relationships of DdCAD-1, glutathione S-transferase fusion proteins containing different DdCAD-1 fragments were expressed and assayed for their GTPase and cell binding activities. These studies revealed that the cell binding activity is dependent on the amino-terminal segment and not the carboxyl-terminal Ca\(^{2+}\)-binding domain and showed additional Ca\(^{2+}\)-binding site(s) within the amino-terminal segment.

The cellular slime mold Dictyostelium discoideum enters the developmental phase of its life cycle upon depletion of nutrients. At an early stage of development, cells undergo chemotactic migration in response to cAMP, leading to the formation of multicellular aggregates called pseudoplasmodia or slugs (for a review, see Ref. 1). Cells at this point are held together by cell-cell adhesion molecules expressed on the cell surface (for a review, see Ref. 2). Depending on environmental conditions, aggregates can either migrate as a slug for a prolonged period of time or undergo rapid differentiation, culminating in the formation of fruiting bodies (1).

Several cell-cell adhesion systems in D. discoideum have been studied in detail in the past two decades (for reviews, see Refs. 2–4). There are two major types of cell-cell contact sites in Dictyostelium. One type is sensitive to low concentrations (1 to 2 mM) of EDTA and the other type resists EDTA dissociation up to a concentration of 15 mM. The EDTA-resistant contact sites, or contact sites A, are expressed at the onset of the cell aggregation stage (5). A cell surface glycoprotein of M\(_r\) 80,000 (gp80) is known to mediate these adhesion sites by homophilic binding (5–9). gp80 cDNAs have been cloned (10, 11), and the deduced amino acid sequence shows limited sequence similarities with the neural cell adhesion molecule NCAM, suggesting that it may belong to the Ig superfamily of cell adhesion molecules (12, 13). An octapeptide sequence near the amino terminus has been identified to be the homophilic binding site of gp80 and it probably interacts in an anti-parallel manner with an identical sequence on an apposing molecule (14, 15). The level of gp80 expression is regulated by both cAMP (16–18) and cell-cell contact (19). There exists at least one other EDTA-resistant cell adhesion system in the post-aggregation stages of Dictyostelium development (3). A membrane glycoprotein of M\(_r\) 150,000 (gp150) has been implicated in Ca\(^{2+}\)-independent cell-cell adhesion in the later stages of development (20–22). gp150 may also play a role in the cell sorting process during morphogenesis (23).

The EDTA-sensitive cell-cell contact sites, or contact sites B, are expressed soon after the initiation of development (24). A concanavalin A-binding protein of M\(_r\) 24,000 (gp24) has been implicated in the EDTA-sensitive cell adhesion system (25). Antibodies raised against gp24 are capable of blocking the EDTA-sensitive contact sites. Subsequent purification and characterization of gp24 has demonstrated that the purified protein possesses intrinsic cell binding activity (26). Since gp24 is a Ca\(^{2+}\)-binding protein and its cell binding activity is sensitive to both EDTA and EGTA, we redesignate this protein as D. discoideum Ca\(^{2+}\)-dependent cell adhesion molecule-1 or DdCAD-1. \(^{125}\)I-Labeled DdCAD-1 binds to cells in a dose-dependent and saturable manner. Furthermore, preincubating cells with anti-DdCAD-1 Fab fragments blocks the binding of labeled DdCAD-1 to cells, suggesting a homophilic mode of interaction. The binding of DdCAD-1 to cells results in the inhibition of EDTA-sensitive cell-cell adhesion and cells are no longer

---

* This work was supported in part by Operating Grant MT-6140 from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† A recipient of a studentship award from the Medical Research Council of Canada.

§ Present address: Department of Medicine, University of Toronto, Toronto, Ontario, M5G 1L6 Canada.

¶ To whom correspondence should be addressed: Charles H. Best Institute, University of Toronto, 112 College St., Toronto, Ontario M5G 1L6, Canada. Tel.: 416-978-8766; Fax: 416-978-8528; E-mail: chi.hung.siu@utoronto.ca.

† The abbreviations used are: gp, glycoprotein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; kb, kilobase pair(s).
capable of reassociating into large aggregates (26). To characterize the structure-function relationships of DdCAD-1, we have done DdCAD-1 cDNAs by immunological screening of αgt11 expression libraries. Here we report the near full-length cDNA sequence of DdCAD-1, which encodes a polypeptide of 23,924 Da. The predicted amino acid sequence exhibits sequence similarities with members of the cadherin family of cell adhesion molecules and with protein S, a Ca2+-binding spore coat protein in Myxococcus xanthus. A substantial amount of DdCAD-1 was localized on the cell surface by immunofluorescence microscopy, although the predicted polypeptide sequence lacks a signal peptide or a transmembrane domain. The carboxyl terminus of DdCAD-1 contains a putative Ca2+-binding motif. However, analysis of GST-DdCAD-1 fusion proteins reveals an internal Ca2+-binding site and shows that the cell binding activity of DdCAD-1 is dependent on the amino-terminal segment and not on the putative Ca2+-binding site in the carboxyl terminus.

**EXPERIMENTAL PROCEDURES**

Cloning of DdCAD-1 cDNA—For immunoscreening, a D. discoideum αgt11 expression library constructed from 3-h cells (a generous gift from Dr. Peter Devreotes of Johns Hopkins University) was used to infect overnight cultures of Escherichia coli strain Y1090 at a density of ~5 × 108 plaque-forming units/180-mm diameter plate. The infected cells, after appropriate dilutions, were then plated on 1.5% LB agar plates with the top 0.7% agar and incubated for 3.5 h at 42°C. Protein expression was induced by overlaying the plate with a nitrocellulose membrane that had been saturated with 10 mM isopropyl β-thiogalactoside. Incubation was continued for another 3.5 h at 37°C or at 4°C overnight. Nitrocellulose membranes were blocked with 5% nonfat dried milk in PBS containing 3 mM sodium azide for 1 h. Incubation with anti-DdCAD-1 IgG prepared from rabbit antiserum (26) was carried out for 1 h. This was followed by incubation with the alkaline phosphatase–conjugated secondary goat anti-rabbit IgG (1:1000 dilution). The filters were washed twice in TTBS (20 mM Tris at pH 7.6, 0.5 M NaCl and 0.5% Tween 20) and twice in TBS (20 mM Tris at pH 7.6 and 0.6 M NaCl). Freshly prepared nitroblue tetrazolium salt solution (6 mg in 0.2 ml of 70% dimethyl formamide) and 5-bromo-4-chloro-3-indolyl phosphate solution (3 mg in 0.2 ml dimethyl formamide) were mixed and diluted in carbonate buffer (0.1 M NaHCO3 and 1 M MgCl2 at pH 9.5) for visualization of positive plaques. Positive recombinant phage plaques were then purified to 100% homogeneity by four cycles of rescoring, and the cDNA inserts were subcloned into pBluescript (Stratagene) for further analysis.

Two partial cDNA clones were obtained by immunoscreening of the 3-h library. They covered almost the complete coding region with the exception of the carboxyl terminus of the protein. A λgt11 library prepared and used to screen another cDNA library prepared from 8 h growing cells was hybridized with the inserts of the two λgt11 clones. Following digestion of λgt11 with SspI, the insert was subcloned into the unique HindIII site of pBluescript (Stratagene). The inserts were thensequencedwithaBio-Radminigelapparatus,ataconstantcurrentof20mAfor1h,usingamM-7-bxolambdainsertcontainingthefDdCAD-1codingsequencebetweenVal-3andLys-213, to the unique EcoRI site of the expression vector pGEX-1X1 (Pharmacia). Then two deletion mutants (GST-DdCAD-1dI and GST-DdCAD-1dII) were generated. The GST-DdCAD-1dI construct, which contained the coding sequence between Val-3 and Thr-203, was generated using the transformer site-directed mutagenesis kit (Stratagene). The GST-DdCAD-1dII construct, which contained the coding sequences between Gin-199 and Lys-213, was subcloned by conformational site reaction fragment into the unique EcoRI site of pGEX-1X1. All constructs were sequenced from both directions to ascertain sequence fidelity and proper orientation. Expression of the fusion protein in transfected cells was determined by immunoblot analysis.

For large scale purification of the fusion proteins, plasmid DNA was used to transform the E. coli strain JM101. Cells were grown overnight at 37°C in the presence of ampicillin (50 μg/ml). The culture was then diluted 10 times and grown at 30°C. After 1.5 h, protein expression was induced by adding isopropyl β-thiogalactoside to a final concentration of 0.5 mM. The culture was harvested at 30°C for 20 h before harvest. Cells were collected at 4°C and the cell pellet was resuspended in sonication buffer (10% glycerol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiotreitol in PBS). Cells were lysed by sonication and the sample was centrifuged at 10,000 rpm for 15 min. The supernatant was then solubilized with 1% Triton X-100, 5 mM MgCl2, and 10% glycerol. Protein concentration was assayed by proteinase K digestion and polyacrylamide gel electrophoresis. After electrophoresis, the fusion proteins were eluted by sonication buffer (10% glycerol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) and stored at −20°C.

**Construction and Expression of GST-DdCAD-1 Fusion Proteins—**The first fusion protein (designated GST-DdCAD-1) was constructed by ligating a 5.7-kb cDNA insert containing the DdCAD-1 coding sequence between Val-3 and Lys-213, to the unique EcoRI site of the expression vector pGEX-1X1 (Pharmacia). Then two deletion mutants (GST-DdCAD-1dI and GST-DdCAD-1dII) were generated. The GST-DdCAD-1dI construct, which contained the coding sequence between Val-3 and Thr-203, was generated using the transformer site-directed mutagenesis kit (Stratagene). The GST-DdCAD-1dII construct, which contained the coding sequences between Gin-199 and Lys-213, was subcloned by conformational site reaction fragment into the unique EcoRI site of pGEX-1X1. All constructs were sequenced from both directions to ascertain sequence fidelity and proper orientation. Expression of the fusion protein in transfected cells was determined by immunoblot analysis.

For large scale purification of the fusion proteins, plasmid DNA was used to transform the E. coli strain JM101. Cells were grown overnight at 37°C in the presence of ampicillin (50 μg/ml). The culture was then diluted 10 times and grown at 30°C. After 1.5 h, protein expression was induced by adding isopropyl β-thiogalactoside to a final concentration of 0.5 mM. The culture was harvested at 30°C for 20 h before harvest. Cells were collected at 4°C and the cell pellet was resuspended in sonication buffer (10% glycerol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiotreitol in PBS). Cells were lysed by sonication and the sample was centrifuged at 10,000 rpm for 15 min. The supernatant was then solubilized with 1% Triton X-100, 5 mM MgCl2, and 10% glycerol. Protein concentration was assayed by proteinase K digestion and polyacrylamide gel electrophoresis. After electrophoresis, the fusion proteins were eluted by sonication buffer (10% glycerol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) and stored at −20°C.

**Construction and Expression of GST-DdCAD-1 Fusion Proteins—**The first fusion protein (designated GST-DdCAD-1) was constructed by ligating a 5.7-kb cDNA insert containing the DdCAD-1 coding sequence between Val-3 and Lys-213, to the unique EcoRI site of the expression vector pGEX-1X1 (Pharmacia). Then two deletion mutants (GST-DdCAD-1dI and GST-DdCAD-1dII) were generated. The GST-DdCAD-1dI construct, which contained the coding sequence between Val-3 and Thr-203, was generated using the transformer site-directed mutagenesis kit (Stratagene). The GST-DdCAD-1dII construct, which contained the coding sequences between Gin-199 and Lys-213, was subcloned by conformational site reaction fragment into the unique EcoRI site of pGEX-1X1. All constructs were sequenced from both directions to ascertain sequence fidelity and proper orientation. Expression of the fusion protein in transfected cells was determined by immunoblot analysis.

For large scale purification of the fusion proteins, plasmid DNA was used to transform the E. coli strain JM101. Cells were grown overnight at 37°C in the presence of ampicillin (50 μg/ml). The culture was then diluted 10 times and grown at 30°C. After 1.5 h, protein expression was induced by adding isopropyl β-thiogalactoside to a final concentration of 0.5 mM. The culture was harvested at 30°C for 20 h before harvest. Cells were collected at 4°C and the cell pellet was resuspended in sonication buffer (10% glycerol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiotreitol in PBS). Cells were lysed by sonication and the sample was centrifuged at 10,000 rpm for 15 min. The supernatant was then solubilized with 1% Triton X-100, 5 mM MgCl2, and 10% glycerol. Protein concentration was assayed by proteinase K digestion and polyacrylamide gel electrophoresis. After electrophoresis, the fusion proteins were eluted by sonication buffer (10% glycerol, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) and stored at −20°C.
45 min. For immunostaining, proteins were transferred electrophoretically onto a nitrocellulose membrane (31). The protein blot was blocked with 5% dried milk and then incubated with an appropriate dilution of the rabbit anti-DdCAD-1 antiserum. The bound antibody was detected using an alkaline phosphatase-conjugated goat-anti-rabbit IgG secondary antibody.

Cell-to-Substratum Attachment Assay—Petri dishes were coated with nitrocellulose solubilized in methanol according to the method of Lagenaur and Lemmon (33), and the dishes were air-dried. 50-μl aliquots of protein samples, containing 5 μg of protein, were placed on 1-cm-diameter spots previously marked on the plastic surface for 1.5 h. After removal of the unbound protein, the dish was blocked with 1% (w/v) bovine serum albumin. The efficiency of protein coating was generally between 30 and 65% of the input protein. However, positive signals were not affected by the differences in coating efficiency, suggesting the amounts of coated protein within the above range were saturating for cell attachment.

Cells were collected at 3 h of development, and aliquots of 50 μl of cell suspension (4 × 10^5 cells/ml) were placed on the protein spots. After a specific period of incubation at room temperature, the spots were washed gently with PBS twice to remove unbound cells. Bound cells were fixed in 3.7% formaldehyde. The number of cells attached per 1-cm^2 spot was determined using a hemocytometer. To determine the effects of each fusion protein on cell reassociation, different amounts of the fusion protein were added to 100-μl cell aliquots. The samples were briefly vortexed, and the assay was carried out as described above.

Cell Adhesion Molecule DdCAD-1 of Dictyostelium—

RESULTS

Molecular Cloning of DdCAD-1 cDNA—Anti-DdCAD-1 IgG derived from a rabbit antisera was used initially to screen a λgt11 expression library prepared from cDNA of 3-h Dictyostelium cells. From this primary screen of −5 × 10^5 plaques, seven positive recombinant phages were isolated. DNA sequencing was carried out for the two longest overlapping cDNA clones, designated DdCAD-1a and DdCAD-1b. Both cDNA inserts contained a single open reading frame. However, sequences coding for the amino-terminal region of the protein were missing. Using a probe derived from DdCAD-1a, we screened 10^5 recombinants from an 8-h λgt11 library. A 1.8-kb cDNA insert designated DdCAD-1c was obtained. Southern blot analysis showed that the insert hybridized with both DdCAD-1a and DdCAD-1b cDNAs (data not shown). Subsequent DNA sequencing revealed a 0.7-kb DdCAD-1 cDNA recombined at its 5’ end with another cDNA sequence. This 0.7-kb fragment contained the entire DdCAD-1 coding sequence (see below). The relationship among these three cDNA inserts and the restriction map of the DdCAD-1 cDNA are shown in Fig. 1A.

Nucleotide Sequence and the Deduced Amino Acid Sequence

FIG. 1. Molecular cloning of DdCAD-1 cDNAs. A, schematic diagram shows the three overlapping cDNA inserts. The DdCAD-1 cDNA inserts DdCAD-1a and DdCAD-1b were obtained by immunological screening of a 3-h λgt11 cDNA library. The cDNA DdCAD-1c was obtained by screening an 8-h expression library using the DdCAD-1a as the probe. The restriction map of the full-length DdCAD-1 cDNA (hatched bar) is shown at the top. B, the nucleotide sequence of the DdCAD-1 cDNA and its deduced amino acid sequences. Both strands of the cDNA inserts were sequenced using the dye-exchange chain termination method (28). The upstream In-frame stop codon (TAA) in the 5’ untranslated region and the consensus polyadenylation signal in the 3’ untranslated region are underlined. The internal CNbr peptide sequences are shown in boldface type. Potential N-glycosylation sites are designated with (●) and the 4 Cys residues are designated with (○). C, amino-terminal sequences of two CNbr-digested DdCAD-1 fragments. Internal fragments of DdCAD-1 were generated by digestion of 50 μg of purified protein with cyanogen bromide overnight at room temperature. Sequences for peptides a and b were determined on a gas-phase microsequencer. Where the identity of the amino acid was not determined, the position was marked with an X. D, hydrophathy plot of the deduced amino acid sequence of DdCAD-1. The hydrophathy plot was generated according to Kyte and Doolittle (35), averaged over windows of 11 residues. Positive values indicate hydrophobicity, while negative values indicate hydrophilicity.
of DdCAD-1—The nucleotide sequence of the DdCAD-1 cDNA consists of an open reading frame of 642 bp (Fig. 1B). The initiation codon ATG is preceded by 5 adenosines and an in-frame stop codon at nucleotide position −6. The reading frame terminates with the stop codon TAA. The coding region has an A + T content of 67%. Analysis of its codon usage reveals that the most frequently used codons have A or T in their third position, typical of Dictyostelium genes.

The predicted polypeptide contains 213 amino acids, with a calculated molecular mass of 23,924 Da (Fig. 1). The identity of the predicted polypeptide also agrees closely with that reported for DdCAD-1 (26). The identity of the DNA clones were further confirmed by two amino acid sequences derived from CNBr peptides of purified DdCAD-1 (Fig. 1, B and C). Both peptide sequences are present within the amino acid sequence deduced from the cDNA, indicating that these cDNA inserts code for DdCAD-1.

The deduced DdCAD-1 polypeptide contains more acidic residues than basic ones, giving rise to a negatively charged protein with a predicted isoelectric point (pI) of 5.30. Four cysteine residues at amino acid positions 16, 44, 52, and 129 provide potential sites for disulfide bond formation which may provide stability for the structure and/or function of DdCAD-1 (Fig. 1B). The deduced protein structure also contains five potential N-glycosylation sites (Fig. 1B).

Although DdCAD-1 has been shown to be a cell adhesion molecule associated with the plasma membrane (26), the Kyte and Doolittle (35) hydrophathy profile of DdCAD-1 is typical of a soluble protein (Fig. 1D). The amino-terminal region is relatively hydrophilic and does not appear to contain a signal peptide sequence. The entire protein has only two short hydrophobic segments at amino acid positions 40–50 and 85–105. The first segment is too short to span a lipid bilayer, and the second segment contains several charged residues and has a relatively low hydrophobicity index to qualify for a transmembrane domain. The hydrophobicity plot of DdCAD-1 thus predicts a soluble cystolic protein, rather than an integral membrane protein.

Secondary structure predictions for DdCAD-1, using the Chou and Fasman algorithm (36, 37), show a high probability that many short segments of the polypeptide have the β-strand conformation, separated by turn structures (data not shown). Only four short regions show a high probability of forming α-helices: Trp-55 to Ile-60, Lys-67 to Leu-71, Lys-127 to Leu-131, and Val-184 to Glu-189.

An interesting feature of the primary structure of DdCAD-1 is the presence of a modest amount of internal sequence homology. The amino acid sequence can be aligned into four homologous segments (Fig. 2). When aligned for maximal homology, the amino acid identity between these segments varies between 20 and 30%. Domain IV is followed by a short sequence, designated domains I to IV (Met-195 and Lys-213), which has no homologous counterparts in the first three domains. However, the carboxyl-terminal region between Met-195 and Asn-210 shows 25% sequence identity with the amino-terminal sequence in domain I. The segment between Thr-198 and Lys-213 shows 56% sequence identity with another segment (Thr-76 to Lys-88) in domain II (Fig. 3B). Interestingly, a putative Ca²⁺-binding sequence has been detected in the carboxyl-terminal region from Asp-200 to Glu-212 (see below). The implication of the internal sequence homology is not clear, but it suggests that DdCAD-1 may be folded into four separate units, followed by a short Ca²⁺-binding tail.

Southern blot analysis indicates the presence of only one gene coding for DdCAD-1. The 32P-labeled cDNA probe hybridized with one major EcoRI fragment of a nuclear DNA digest (Fig. 3). Although the cDNA sequence shows the presence of a HindI site, only one major band was observed in the HindI digest, suggesting that the two HindI fragments were of similar sizes. When the genomic DNA was digested with both HindI and EcoRI, two major fragments were observed. Digestion with HaeIII yielded one major band and a minor band at −0.6 kb, in agreement with the presence of a HaeIII site near the 3′-end of the coding sequence. Two weakly labeled bands were also visible, and they may represent other genes that share sequence similarities with the DdCAD-1 gene.

Subcellular Localization of DdCAD-1—DdCAD-1 is expected to be present on the cell surface to function as a cell adhesion molecule. However, the deduced amino acid sequence suggests that DdCAD-1 is a soluble cytosolic protein. To resolve this issue, immunofluorescence labeling was used to investigate the subcellular localization of DdCAD-1. Using laser scanning confocal microscopy, we examined cells in the initial stages of development prior to the onset of chemotactic migration. The cytoplasm showed diffuse staining of DdCAD-1, consistent with the notion that DdCAD-1 is a soluble protein in the cytoplasm. However, intense staining was also observed in the peripheral regions of the cell (Fig. 4a). When cell pairs were analyzed, an increase in DdCAD-1 staining intensity in the intercellular contact area was evident, indicating a higher concentration of DdCAD-1 in cell junctions (Fig. 4c).

To demonstrate that at least a portion of the peripheral labeling was due to the DdCAD-1 molecules present on the cell surface, capping of DdCAD-1 was induced by incubating cells at room temperature with primary antibodies for 20 min, followed by secondary antibodies for 10 min, before fixation for laser scanning confocal microscopy. DdCAD-1 patches and caps were observed on most cells, whereas a more or less uniform
expression of DdCAD-1 thus corresponds closely to the temporal level, at 3, 6, and 9 h of development, respectively. The relative levels of DdCAD-1 expression at different stages of development were examined using Western blot (Fig. 6). The protein levels of DdCAD-1 mRNA occurred by 9 h of development. The relative accumulation was observed at 6 h and the level increased 4-fold by 3 h. Maximal level of DdCAD-1 mRNA accumulation was observed at 6 h and the level increased >15-fold over the level at 0 h. A small decline in the level of DdCAD-1 mRNA occurred by 9 h of development. The relative levels of DdCAD-1 expression at different stages of development were examined using Western blot (Fig. 6B). The protein level of DdCAD-1 showed a rapid increase between 3 and 6 h, and it peaked at 9 h of development. Levels of DdCAD-1 expression increased by 2.5, 7, and 10-fold, as compared to the 0-h level, at 3, 6, and 9 h of development, respectively. The expression of DdCAD-1 thus corresponds closely to the temporal expression of the EDTA-sensitive cell-cell binding sites (25, 38).

Sequence Similarities between DdCAD-1 and Other Cell Adhesion Molecules—A sequence homology search was carried out to identify molecules that might be homologous to DdCAD-1. DdCAD-1 exhibits sequence similarity with protein S of M. xanthus and members of the cadherin family (Fig. 7). Protein S is a Ca^{2+}-binding spore coat protein (39, 40), which shows a relatively high degree of sequence similarity with E-cadherin (41). Similar to DdCAD-1, protein S consists of four internally homologous motifs (42, 43). Domains I and II of DdCAD-1 can be aligned with motifs 1 and 2 of protein S with 70% sequence similarity. However, large gaps have to be introduced in the alignment of the next two domains. Overall, DdCAD-1 shows 19% sequence identity and 43% sequence similarity with protein S (Fig. 7A).

DdCAD-1 shows a slightly higher degree of sequence similarity with members of the cadherin family (Fig. 7C). Specifically, domains II and III can be aligned with the first extracellular repeat of E-cadherin, with 28% sequence identity. When the conservative substitutions in amino acids are taken into account, there is 48% similarity between the two sequences. The unique Pro-rich region between amino acids 141 and 148 of DdCAD-1 marks the end of its similarity with the first cadherin repeat. This is followed by sequences in DdCAD-1 domain IV which also aligns well with the first half of the second repeat of the cadherins. The first cadherin repeat contains two short \( \alpha \)-helices and seven \( \beta \)-strands. Analysis using the Chou and Fasman algorithms (36, 37) indicates that segments in DdCAD-1 that align with the seven \( \beta \)-strands of E-cadherin also show a relatively high propensity for the \( \beta \)-strand conformation.

Residues involved in binding Ca^{2+} have been identified for both protein S and E-cadherin. They do not reside within a small oligopeptide sequence as in the case of the classic EF-hand motif. Instead residues from different parts of the protein come together to form negatively charged pockets that bind Ca^{2+} (see highlighted residues in Fig. 7, A and C). Our alignments show that frequently similar, although not identical, residues are present in similar locations in DdCAD-1.

Several short segments of DdCAD-1 exhibit significant similarities to the human IgA-1 chain \( \alpha \) and the LFA-1 \( \alpha \) subunit (Fig. 7B). The Pro-rich region of DdCAD-1, between residues Pro-139 and Pro-146, shows a high degree of sequence identity (50%) with the hinge region of the human IgA-1 chain \( \alpha \) (Fig. 7B). This short segment contains four prolines in both proteins, suggesting that this region may serve a hinge mechanism in DdCAD-1. Between amino acid positions 44 and 52 are two of the four Cys residues of DdCAD-1 and this sequence shows 56% sequence identity with the integrin LFA-1 \( \alpha \). Additional se-

**Fig. 3. Southern blot analysis of nuclear DNA.** Nuclear DNA was isolated from KAX3 cells and then digested with different restriction enzymes: a, EcoRI; b, HindII; c, HaelII; and d, EcoRII and HindI. Restriction fragments were separated in a 1% agarose gel, blotted onto a nitrocellulose membrane, and hybridized to the \(^{32}P\)-labeled DdCAD-1 cDNA probe. Molecular markers using \( \lambda \) DNA are indicated on the left.

**Fig. 4. Subcellular localization of DdCAD-1 by immunofluorescence staining.** KAX3 cells were developed under submerged culture conditions on coverslips. Cells were fixed with 3.7% formaldehyde in 17 mM phosphate buffer, pH 6.4, for 15 min, permeabilized with cold methanol (−20 °C) for 5 min, and then stained with anti-DdCAD-1 antibody, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody, a, an X/Y image of a single cell at 6 h of development showing cytoplasmic and enhanced peripheral staining of DdCAD-1; b, a 6-h control cell stained with preimmune serum; and c, an X/Z image showing an increase in DdCAD-1 staining intensity in the intercellular contact region between two cells at 12 h of development. Bar = 5 \( \mu \)m.
Sequence similarity is noted between the segment from Pro-63 to Gly-73 in DdCAD-1 and the membrane-proximal region of LFA-1α.

Sequence Similarity with Ca²⁺-binding Proteins—A possible calcium-binding sequence is found near the carboxyl terminus: 200DDNTSFIFNLNSE212 (Fig. 8). This segment of DdCAD-1 shows a significant degree of similarity to the loop region of the EF-hand helix-loop-helix structure in many calcium-binding proteins (44). The critical residues involved in liganding Ca²⁺ are designated 1X, 1Y, 1Z, 2X, 2Y, and 2Z. The frequencies at which each of these critical residues within the DdCAD-1 putative Ca²⁺-binding sequence are found in other Ca²⁺-binding proteins are 98% (D), 24% (N), 22% (S), 2.4% (I), 6% (N), and 0% (S) at positions +X, +Y, +Z, −X, −Y, and −Z, respectively (41). The most commonly occurring residues are D (98%), D (73%), D (56%), F (16%), D (33%), and E (86%) at positions +X, +Y, +Z, −X, −Y, and −Z, respectively (41). Although DdCAD-1 contains Ser instead of Glu at the 2Z position, it should be noted that a Glu residue is present immediately after the Ser residue. It is therefore likely that this carboxyl-terminal sequence of DdCAD-1 may serve as a Ca²⁺-binding domain.

Expression and Purification of Fusion Proteins—DdCAD-1 is a Ca²⁺-binding protein and its cell binding activity is dependent on Ca²⁺ (26). As a first step to investigate the structure/function relationships of DdCAD-1, we examined the significance of the putative Ca²⁺-binding loop in the carboxyl-terminal region of DdCAD-1. GST-fusion proteins containing
different DdCAD-1 fragments were constructed using the expression vector pGEX-1Xl (Fig. 9A) and they were subjected to different binding assays. E. coli cells transformed with these recombinant plasmids produced soluble fusion proteins with the glutathione S-transferase fused to the amino terminus of the DdCAD-1 polypeptide. GST-DdCAD-1 contained a DdCAD-1 polypeptide spanning between Val-3 and Lys-213. The fusion protein GST-DdCAD-1dl contained a DdCAD-1 fragment between Val-3 and Thr-203. The insert consisting of the putative Ca\(^{2+}\)-binding segment of DdCAD-1 (Gln-199 to Lys-213) was used to generate GST-DdCAD-1dl. These three fusion proteins were purified by passing through a glutathione-Sepharose 4B column and the purified proteins were analyzed by SDS/polyacrylamide gel electrophoresis (Fig. 9B). They migrated with apparent molecular sizes comparable to the predicted sizes of the fusion proteins. When subjected to immunoblot analysis, all three GST fusion proteins reacted positively with the rabbit anti-DdCAD-1 antiserum (Fig. 9C), and they did not react with the preimmune serum (data not shown).

Effects of Fusion Proteins on Cell-Cell Adhesion—Soluble DdCAD-1 is known to bind to cells and inhibit Ca\(^{2+}\)-dependent cell-cell adhesion (26). We tested whether purified GST-DdCAD-1 fusion proteins still retained a functional cell binding sites. Those that retained the cell binding activity were expected to block cell-cell adhesion mediated by the membrane-associated DdCAD-1 molecules. Cells were developed for 3 h, dissociated mechanically into single cells, and then allowed to reassociate in different concentrations of the GST fusion proteins. Cell reassociation was significantly inhibited when the cell cohesion assay was carried out in the presence of GST-DdCAD-1 (Fig. 10A). The level of inhibition by GST-DdCAD-1 was similar to that of native DdCAD-1. The inhibitory effects of GST-DdCAD-1 were dose-dependent and maximal inhibition was achieved at a protein concentration of ~0.1 \(\mu\)M (Fig. 10B). GST by itself did not exert any deleterious effect on cell reassociation. These results indicate that the cell binding site in GST-DdCAD-1 was fully functional.

When the two GST-fusion proteins containing partial deletions of DdCAD-1 were assayed, GST-DdCAD-1dl was found to inhibit cell reassociation to an extent similar to that of GST-DdCAD-1 (Fig. 11). In contrast, GST-DdCAD-1dII had no appreciable effect on cell reassociation. The morphology of cell

---

**FIG. 8.** Sequence similarity between DdCAD-1 and Ca\(^{2+}\)-binding proteins. Sequence of a putative DdCAD-1 calcium-binding loop between amino acid positions 200 and 213 is aligned with those of chicken cCM1 calmodulin, rat parvalbumin A, chicken myosin skeletal L2, and platelet membrane glycoprotein (44). The positions of the residues involved in liganding Ca\(^{2+}\) are indicated as +X, +Y, +Z, -X, -Y, and -Z. Residues present in both the DdCAD-1 sequence and the known Ca\(^{2+}\)-binding loop sequences are shown in boldface type.

**FIG. 9.** Construction and Expression of GST-DdCAD-1 fusion proteins. A, schematic drawings showing the three GST-DdCAD-1 fusion protein constructs. DdCAD-1 cDNA inserts were cloned into the unique EcoRI site of pGEX-1Xl expression vector and the DdCAD-1 fragments were fused in frame to the carboxyl-terminal region of GST. B, gel profiles and immunoblots of GST-DdCAD-1 fusion proteins. GST fusion proteins were purified and analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue (lanes a, b, and c). The proteins were transferred to a nitrocellulose membrane and stained with anti-DdCAD-1 antibody (lanes d, e, and f). Lanes a and f, GST-DdCAD-1; lanes b and e, GST-DdCAD-1dl; lanes c and d, GST-DdCAD-1dII.
aggregates formed in the absence of these fusion proteins was also observed using phase microscopy. Aggregates formed in samples containing GST or GST-DdCAD-1dII were about 10 to 30 cells in size, whereas aggregates formed in the presence of native DdCAD-1, GST-DdCAD-1, or GST-DdCAD-1dI were mostly much smaller, composed of only 3–10 cells (data not shown). Although GST is capable of forming dimers in solution (46), nonspecific agglutination due to GST was minimal (Fig. 10A) and the fusion proteins were just as effective as purified DdCAD-1 in the inhibition of cell reassociation (Figs. 10 and 11).

The cell binding activity of these recombinant DdCAD-1 proteins was further evaluated using the cell-to-substratum adhesion assay. Cells expressing DdCAD-1 were allowed to attach to plastic surfaces coated with the GST-fusion proteins. The amount of cells bound to substrate-coated GST-DdCAD-1 or GST-DdCAD-1dI was ~2.5-fold higher than that bound to the GST substrate, and the assay was effectively inhibited by soluble DdCAD-1 and anti-DdCAD-1 Fab (Fig. 12). In contrast, GST-DdCAD-1dII yielded only the background level of cell attachment.

Binding of Ca$^{2+}$ to Fusion Proteins—Since the cell binding activity of DdCAD-1 is dependent on Ca$^{2+}$, it is likely that the DdCAD-1 fragment in GST-DdCAD-1dI contained Ca$^{2+}$-binding site(s). To determine whether the two DdCAD-1 fragments in GST-DdCAD-1dI and GST-DdCAD-1dII had Ca$^{2+}$-binding activity, $^{45}$Ca$^{2+}$ overlay assays were carried out. Protein samples were blotted onto nitrocellulose membrane using a slot blot apparatus and the blot was equilibrated with $^{45}$Ca$^{2+}$, followed by autoradiography. Calmodulin was included as a positive control and it gave a strong positive signal, while GST did not bind significant amounts of $^{45}$Ca$^{2+}$. All three GST-fusion proteins bound $^{45}$Ca$^{2+}$ (Fig. 13A). Relatively strong signals were obtained with GST-DdCAD-1 and GST-DdCAD-1dI.
while a weaker signal was obtained with GST-DdCAD-1dII. The specificity of the interactions between these GST-fusion proteins and Ca\(^{2+}\) was demonstrated in competition experiments, where a 1500-fold excess of unlabeled Ca\(^{2+}\) was included in the assay (Fig. 13B). In all cases, \(^{45}\)Ca\(^{2+}\) binding was displaced by the excess cold Ca\(^{2+}\).

**DISCUSSION**

We have cloned and sequenced cDNAs encoding the cell-cell adhesion molecule DdCAD-1 in *D. discoideum*. An earlier attempt of cloning DdCAD-1 resulted in the isolation of a pair of tandemly repeated genomic sequences (47), which share no similarity to the clones described in this report. These genes are believed to be pseudogenes (49, 50), and there is no other ATG sequence between the RNA transcription site and the initiation codon.

The deduced amino acid sequence of DdCAD-1 reveals several interesting features that are consistent with the biochemical characteristics of DdCAD-1. First, the predicted mature polypeptide has a Ser residue at its amino terminus and an amino-terminal Ser in cytosolic proteins is often acetylated (51), consistent with our finding that the amino terminus of DdCAD-1 is blocked (26). Second, nonreduced DdCAD-1 migrates faster with an apparent molecular weight of 22,000, suggesting the presence of disulfide bridges which can maintain a more compacted structure even in the presence of SDS (26). This is consistent with the presence of four Cys residues in the deduced polypeptide, which can potentially form intramolecular disulfide bonds.

Another interesting feature of DdCAD-1 is the apparent lack of a signal peptide or transmembrane domain in the deduced amino acid sequence. The hydrophathy plot of DdCAD-1 is typical of soluble proteins. However, a substantial amount of DdCAD-1 is present on the cell surface and an enrichment of DdCAD-1 in intercellular contacts is also observed, consistent with the role of a cell-cell adhesion molecule. These observations thus raise the question of how DdCAD-1 is transported and anchored to the cell surface. Current studies in our laboratory suggest that DdCAD-1 is transported to the plasma membrane via contractile vacuoles (3), and that the cell surface association of DdCAD-1 may be mediated by its association with an integral membrane protein (52).

DdCAD-1 shows sequence similarities with protein S and members of the cadherin family, suggesting that both protein S and DdCAD-1 are primitive members of the cadherin family and that DdCAD-1 may be an intermediate form between protein S and the vertebrate cadherins. Interestingly, domains II and III of DdCAD-1 align with a relatively high degree of sequence similarity with the first cadherin repeat of E-cadherin and domain IV can be aligned with the amino-terminal segment of the second cadherin repeat (53). The three-dimensional structure of the cadherin repeated domain has been solved recently (54, 55). The structure shows a remarkable similarity to the Ig fold (56, 57), with a putative adhesive interface containing the highly conserved His-Ala-Val sequence (58, 59). Since all seven β-strands of the cadherin domain align with regions of DdCAD-1 that also show a high propensity for β-strand formation, we speculate that DdCAD-1 may also adopt a similar folded structure. If this is the case, the predicted β segment consisting of the C, F, and G β-strands may constitute the face involved in DdCAD-1 homophilic interactions.

The Ca\(^{2+}\)-binding site of cadherin is located on the opposite side of the putative adhesive site. It involves the linkage region between the extracellular repeat 1 and 2. Three acidic residues from different regions of the first domain and the Asp-Ala-Asp sequence in the second domain constitute the acidic pocket for binding Ca\(^{2+}\) (54, 55). The aligned sequences show that identical residues are found in two out of four of these positions in DdCAD-1, but their role in Ca\(^{2+}\)-binding has not been shown. The binding of Ca\(^{2+}\) to cadherin results in an extended rod-shaped structure (60), thus allowing homophilic interactions between two apposing molecules (55). It is therefore likely that the binding of Ca\(^{2+}\) will also lead to conformational changes in DdCAD-1. Indeed, DdCAD-1 shows a small shift in gel mobility upon binding with Ca\(^{2+}\), suggesting a change in protein conformation (26).

Since the cell binding activity of DdCAD-1 is sensitive to both EDTA and EGTA, Ca\(^{2+}\)-binding may play a role in the activation of the cell binding site (26). There are at least two Ca\(^{2+}\)-binding sites in DdCAD-1. One site is located in the carboxyl-terminal segment of DdCAD-1, which shows sequence homology to the loop region of the classic EF-hand motif (44, 61). It is remarkable that these last 15 amino acids at the carboxyl terminus alone can confer Ca\(^{2+}\)-binding activity to the GST fusion protein. However, this Ca\(^{2+}\)-binding site is apparently not required for the cell binding activity of DdCAD-1. It remains to be determined whether this site plays a role in anchoring DdCAD-1 to other membrane components. There is at least one additional Ca\(^{2+}\)-binding site in the amino-terminal portion of the molecule. Since no obvious Ca\(^{2+}\)-binding motif has been detected, it is likely that the Ca\(^{2+}\)-binding pocket is constituted by residues from different regions, similar to the case of E-cadherin and protein S. The cell binding activity is likely to be influenced by this Ca\(^{2+}\)-binding site. Further mutational studies and structural analysis will be required to define the relationship between the Ca\(^{2+}\)-binding site and the cell binding activity of DdCAD-1.

In addition to structural similarities, DdCAD-1 resembles cadherin in many aspects of its function. Both DdCAD-1 and cadherin mediate the Ca\(^{2+}\)-dependent type of cell-cell adhesion. Compaction of preimplantation embryos is prevented when they are treated with antibodies against E-cadherin (62). Similarly, treatment with EDTA or Carnitine will inhibit the

---

2 C. Yang and C.-H. Siu, manuscript in preparation.

3 H. Sesaki and C.-H. Siu, manuscript in preparation.
Ca	extsuperscript{2+}-dependent cell binding sites of Dictyostelium cells, resulting in the formation of loose aggregates and the loss of the compacted morphology (63, 64). Cells developed in the presence of antibodies against DdCAD-1 are blocked at the aggregation stage and cannot undergo further morphogenesis (65).

Cell adhesion molecules are known to be important regulators that can control cell polarity, morphogenesis, tissue integrity, and metastasis (66–71). In addition to intercellular adhesion, there is increasing evidence that the classic cadherins are involved in signal transduction (72). In Dictyostelium, several recent reports have shown that cell-cell contact can elicit cAMP secretion and modulate CAMP signaling in Dictyostelium (73–75). Recent studies in our laboratory have demonstrated a role for cell-cell contact in a CAMP-dependent pathway that regulates gene expression, as well as in the CAMP signal relay system (19). Inhibition of DdCAD-1-mediated cell-cell adhesion sites prevents the CAMP-stimulated increase in gp80 expression and treatment of cells with exogenous CAMP pulses cannot overcome this inhibition (19, 52). Exactly how cell-cell contacts mediated by DdCAD-1 initiate or influence a signaling pathway is not known. We have recently found that DdCAD-1 forms a complex with several membrane and cytoplasmic components, which potentially may have a role in the dynamics of the cytoskeleton as well as signal transduction. Future studies on the interactions between DdCAD-1 and these components will be important in our understanding of the role of DdCAD-1 in signal transduction and morphogenetic events.

Acknowledgments—We thank Dr. Peter Devreotes of Johns Hopkins University for providing a sample of a 3-h cDNA library, Drs. D. H. MacLennan and R. Reithmeier for advice and a critical appraisal of the manuscript, Drs. M. Ikura and W. F. Loomis for suggestions and discussion, and T. Y. Lam for expert assistance.

REFERENCES

1. Loomis, W. F. (1993) Curr. Top. Dev. Biol. 28, 1–46
2. Gerisch, G. (1980) Curr. Top. Dev. Biol. 14, 234–270
3. Siu, C.-H. (1990) Bioassays 12, 357–362
4. Fontana, D. R. (1995) In The Principles of Cell Adhesion (Richardson, P. D., and Steiners, M. eds) pp. 63–86, CRC Press, Boca Raton, FL
5. Beug, H., Katz, F., and Gerisch, G. (1973) J. Cell Biol. 66, 674–658
6. Muller, K. and Gerisch, G. (1978) Nature 274, 445–447
7. Brodie, C., Klein, C., and Swierkosz, J. (1983) Cell 32, 1115–1123
8. Siu, C.-H., Lam, T. Y., and Cho, A. H. C. (1985) J. Biol. Chem. 260, 16030–16036
9. Siu, C.-H., Cho, A. H. C. (1987) J. Biol. Chem. 262, 4753–4767
10. Wong, L. M. and Siu, C.-H. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 4248–4252
11. Matsunaga, T. and Mori, N. (1987) Exp. Cell Res. 175, 22–28
12. Matsunaga, T., Pickering, G., Geltosky, J., and Siu, C.-H. (1981) Differentiation 24, 223–228
13. Siu, C.-H., Lam, T. Y., and Cho, A. H. C. (1988) J. Cell Biol. 107, 1835–1843
14. Siu, C.-H., Garrod, D. (1972) Exp. Cell Res. 72, 588–591