

# The Cell Adhesion Molecule DdCAD-1 in *Dictyostelium* Is Targeted to the Cell Surface by a Nonclassical Transport Pathway Involving Contractile Vacuoles

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**Abstract.** DdCAD-1 is a 24-kD  $\text{Ca}^{2+}$ -dependent cell-cell adhesion molecule that is expressed soon after the initiation of development in *Dictyostelium* cells. DdCAD-1 is present on the cell surface as well as in the cytosol. However, the deduced amino acid sequence of DdCAD-1 lacks a hydrophobic signal peptide or any predicted transmembrane domain, suggesting that it may be presented on the cell surface via a nonclassical transport mechanism. Here we report that DdCAD-1 is transported to the cell surface via contractile vacuoles, which are normally involved in osmoregulation. Immunofluorescence microscopy and subcellular fractionation revealed a preferential association of DdCAD-1 with contractile vacuoles. Proteolytic treatment of isolated contractile vacuoles degraded vacuole-associated calmodulin but not DdCAD-1, demonstrating that DdCAD-1 was present in the lumen. The use of hyperosmotic conditions that suppress contractile vacuole ac-

tivity led to a dramatic decrease in DdCAD-1 accumulation on the cell surface and the absence of cell cohesiveness. Shifting cells back to a hypotonic condition after hypertonic treatments induced a rapid increase in DdCAD-1-positive contractile vacuoles, followed by the accumulation of DdCAD-1 on the cell membrane. 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, a specific inhibitor of vacuolar-type  $\text{H}^{+}$ -ATPase and thus of the activity of contractile vacuoles, also inhibited the accumulation of DdCAD-1 on the cell surface. Furthermore, an in vitro reconstitution system was established, and isolated contractile vacuoles were shown to import soluble DdCAD-1 into their lumen in an ATP-stimulated manner. Taken together, these data provide the first evidence for a nonclassical protein transport mechanism that uses contractile vacuoles to target a soluble cytosolic protein to the cell surface.

**T**HE cellular slime mold *Dictyostelium discoideum* transits from the solitary amoeboid state to an organized multicellular structure during development. This process is initiated in cells upon the depletion of nutrients, leading to the expression of many developmentally regulated genes and the chemotactic migration of cells in response to extracellular cAMP. Cells stream in concentric rings and/or spirals toward aggregation centers, giving rise to multicellular entities called pseudoplasmodia or slugs. The migrating slugs eventually culminate in the formation of fruiting bodies consisting of primarily spores and stalk cells (for review see Loomis, 1975).

Multicellularity during development is maintained by the expression of cell-cell adhesion molecules, which fall into two broad categories based on their sensitivity to

EDTA (for reviews see Gerisch, 1980; Siu et al., 1988; Siu, 1990; Fontana, 1995; Bozzaro and Ponte, 1995). There are two types of EDTA-sensitive cell adhesion sites. The EDTA/EGTA-sensitive cell adhesion sites, also known as contact sites B, are mediated by the  $\text{Ca}^{2+}$ -dependent cell adhesion molecule gp24/DdCAD-1 (Knecht et al., 1987; Brar and Siu, 1993), while the EDTA-sensitive/EGTA-resistant sites are probably mediated by a  $\text{Mg}^{2+}$ -dependent cell adhesion molecule (Fontana, 1993). The molecular nature of the latter sites is not yet known. Both types of adhesion sites are responsible for cell-cell interactions in the early stages of development. Coinciding with the aggregation stage is the rapid accumulation of the cell adhesion molecule gp80, which mediates the EDTA-resistant cell adhesion sites or contact sites A (Muller and Gerisch, 1978; Siu et al., 1985; Kamboj et al., 1988, 1989). In postaggregation stages, the EDTA-resistant adhesion sites are mediated by the membrane glycoprotein gp150 (Geltosky et al., 1979; Siu et al., 1983; Gao et al., 1992).

DdCAD-1 is expressed by cells soon after the initiation

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of development (Knecht et al., 1987). Antibodies raised against gel-purified DdCAD-1 specifically inhibit the EDTA/EGTA-sensitive cell-cell adhesion sites and block development (Loomis, 1988). We have purified DdCAD-1 to homogeneity and demonstrated that labeled soluble DdCAD-1 binds to cells in an EDTA/EGTA-sensitive manner (Brar and Siu, 1993). Binding of DdCAD-1 to cells is prevented when cells are precoated with anti-DdCAD-1 antibodies, consistent with a homophilic mode of interaction. In addition, binding of DdCAD-1 to cells inhibits cell reassociation, indicating that it contains only a single cell binding site.

Recent cloning of the DdCAD-1 cDNA predicts a protein of 23,924 daltons (Wong et al., 1996). The deduced amino acid sequence of DdCAD-1 shows significant sequence similarities with members of the cadherin family, and it contains a  $\text{Ca}^{2+}$ -binding motif residing in the carboxy-terminal region. Indeed,  $\text{Ca}^{2+}$  overlay experiments have shown that DdCAD-1 is a  $\text{Ca}^{2+}$ -binding protein with multiple binding sites (Brar and Siu, 1993; Wong et al., 1996). It is therefore conceivable that DdCAD-1 is a primitive member of the cadherin superfamily and it may mediate cell-cell adhesion in a manner similar to that of cadherins (Shapiro et al., 1995; Nagar et al., 1996). Another novel feature of the predicted sequence is that it lacks an amino-terminal hydrophobic signal peptide or a transmembrane domain, suggesting that DdCAD-1 is a soluble protein. Consistent with this observation, both subcellular fractionation and immunofluorescence microscopy have revealed a predominant cytoplasmic localization of DdCAD-1, indicating that 60–80% of DdCAD-1 is soluble (Brar and Siu, 1993; Sesaki and Siu, 1996). However, IgG binding and capping experiments clearly demonstrate that a substantial amount of DdCAD-1 is present on the cell surface (Brar and Siu, 1993; Wong et al., 1996). Interestingly, DdCAD-1 undergoes rapid translocation from the cytoplasm to the plasma membrane in the preaggregation stage of development (Sesaki and Siu, 1996), and then it becomes concentrated on filopodial structures and in cell-cell contact regions. These observations thus raise the question of how DdCAD-1 is transported and anchored to the cell surface.

In this report we present morphological and biochemical evidence that DdCAD-1 is transported to the cell surface from the cytosol via contractile vacuoles, which is known so far to function exclusively in osmoregulation in cells. Furthermore, we show that isolated contractile vacuoles selectively take up soluble DdCAD-1 into their lumen in a cell-free system. Our results demonstrate, for the first time, a protein targeting function for contractile vacuoles and a novel nonclassical protein transport mechanism.

## Materials and Methods

### Cell Strains and Culture Conditions

NC4 cells were cultured on agar dishes in association with *Klebsiella aerogenes* as the food source (Sussman, 1987). Cells were grown to a density of  $10^8$  cells per 100-mm-diam plate and then collected for experiments. Bacteria were removed by differential centrifugation. The axenic strain KAX3 was cultured in HL-5 medium (Sussman, 1987). The KAX3 cells overproduced DdCAD-1 and provided excellent signals for immunofluorescence microscopy. These cells were used in most experiments. For de-

velopment under submerged conditions, cells were washed three times with 17 mM phosphate buffer, pH 6.4, and resuspended at  $2 \times 10^6$  cells per ml in the same buffer. Approximately  $10^6$  cells were deposited on a coverslip coated with 0.1% poly-L-lysine, and 0.4 ml of buffer was removed after 10 min. Coverslips were placed in a moist chamber, and development of these cells was carried out at room temperature. For development in suspension, cells were suspended at  $1\text{--}1.5 \times 10^7$  cells per ml in 17 mM phosphate buffer, pH 6.4, and rotated at 180 rpm on a platform shaker at room temperature.

### Laser Scanning Confocal Microscopy

Cells were fixed according to Fukui et al. (1987) and then processed for laser scanning confocal microscopy (LSCM)<sup>1</sup> as described previously (Sesaki and Siu, 1996). Cells were developed on coverslips for different time periods and then fixed with 3.7% formaldehyde in 17 mM phosphate buffer for 15 min at room temperature, followed by permeabilization with cold methanol ( $-20^\circ\text{C}$ ) containing 1% formaldehyde for 5 min. Nonspecific binding was blocked by incubation with 1% (wt/vol) BSA in PBS for 10 min. Samples were incubated with the anti-DdCAD-1 antiserum (1:200 dilution in PBS containing 0.1% BSA) for 1 h, washed three times with PBS containing 0.05% Tween-20, and then stained with FITC-conjugated goat anti-rabbit IgG (1:300 dilution) for 1 h. For double immunofluorescence labeling, anti-gp80 mAb 80L5C4 (1:100) (Siu et al., 1985), anti-calmodulin mAb mixture, 6D4, 1F11, and 2D1 (Sigma Chemical Co., St. Louis, MO) (1:100 dilution) (Zhu and Clarke, 1992), and anti- $\text{H}^+$ -ATPase mAb N2 (1:50) (Fok et al., 1993) were used, followed by Texas red-conjugated goat anti-mouse IgG (1:300 dilution). For double immunostaining for DdCAD-1 and the *Dictyostelium* lysosomal enzyme  $\alpha$ -mannosidase, cells were developed for 6 h in suspension and then placed on coverslips. After a 10-min incubation at room temperature, cells were fixed with 3.7% formaldehyde for 2 h, permeabilized with 1% saponin in PBS, and then stained with anti-DdCAD-1 antibody and anti- $\alpha$ -mannosidase mAb (2H9) (1:100) (Bush and Cardelli, 1989). Coverslips were mounted in PBS containing 80% glycerol, 0.2% *p*-phenylenediamine, and 2.5% 1,4-diazabicyclo-[2,2,2]-octane. Images were acquired using an MRC 600 confocal imaging system (Bio Rad Laboratories, Hercules, CA) on an Optiphot microscope (Nikon, Tokyo, Japan) equipped with a 40 $\times$  objective. Alternatively, an Axiovert 135 inverted microscope equipped with a 63 $\times$  Neofluar objective and LSM 410 confocal attachment was used (Carl Zeiss, Inc., Thornwood, NY).

### Subcellular Fractionation

Subcellular fractionation was performed following the method of Nolte and Steck (1994) with minor modifications. After development for 12 h in liquid culture, KAX3 cells ( $1.5 \times 10^8$  cells in 10 ml) were disrupted at room temperature in GMC buffer (5 mM glycine-NaOH, 1 mM  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , pH 8.5) using membrane filters with 5- $\mu\text{m}$ -diam pores (Millipore Corp., Bedford, MA). A postnuclear supernatant (1 ml), which was prepared by centrifugation of homogenates at 1,500 rpm for 5 min at  $4^\circ\text{C}$ , was layered on top of a 12-ml 30–60% (wt/vol) sucrose gradient prepared in GMC buffer. Gradients were centrifuged at 33,000 rpm using an SW40 rotor (Beckman Instruments, Inc., Palo Alto, CA) at  $4^\circ\text{C}$  for 3 h. Fractions (0.72 ml each) were collected from the top. Distribution of organelles in gradients was determined using specific enzyme markers (Padh et al., 1989; Nolte et al., 1991): alkaline phosphatase for contractile vacuoles, vanadate-sensitive  $\text{H}^+$ -ATPase for the plasma membrane,  $\text{F}_1\text{F}_0$ -ATPase for mitochondria, and acid phosphatase for lysosomes. To determine the distribution of DdCAD-1, 0.5 ml of each fraction was diluted with 2.5 ml of GMC buffer containing 30 mM NaOH, incubated on ice for 30 min, and centrifuged at 33,000 rpm for 1 h at  $4^\circ\text{C}$  (Luna et al., 1981). Pelleted membrane-associated proteins were subjected to SDS-PAGE (Laemmli, 1970), transferred onto nitrocellulose membrane, and stained with anti-DdCAD-1 antibody.

For proteolytic digestion, proteinase K was added to give a final concentration of 100  $\mu\text{g}/\text{ml}$  in 100  $\mu\text{l}$  of the contractile vacuole fraction in the presence or absence of 1% Triton X-100 and then was incubated at  $37^\circ\text{C}$  for 2 h. After the addition of PMSF at 2 mM, samples were boiled for 10 min and subjected to SDS-PAGE, followed by Western blot analysis using anti-DdCAD-1 and anti-calmodulin antibodies.

1. *Abbreviations used in this paper:* GST, glutathione-S-transferase; LSCM, laser scanning confocal microscopy; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

## Quantification of DdCAD-1 on the Cell Surface

All steps were carried out on ice unless noted otherwise. After development for a specific time period,  $7.5 \times 10^6$  cells were resuspended in 1 ml of 0.1% BSA, 2 mM EGTA, and 17 mM phosphate buffer, pH 6.4, and rotated at 180 rpm for 10 min to block nonspecific binding sites. Next, anti-DdCAD-1 IgG (8.2  $\mu$ g) was added to the cell suspension and incubated for 15 min. Cells were washed three times using the same buffer solution and then incubated with HRP-conjugated goat anti-rabbit IgG (1:50,000 dilution) for 15 min on a platform shaker. After three washes, cells were resuspended in 0.5% Triton X-100, 0.5 mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), 0.05% H<sub>2</sub>O<sub>2</sub>, and 100 mM citrate-NaOH, pH 4.0, and then incubated for 15 min at room temperature. Samples were clarified by centrifugation and absorbance of the supernatant was measured at OD<sub>405</sub>.

## Inhibition of Contractile Vacuole Activity by Hypertonic Treatments and 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole

For hypertonic treatments, NC4 cells were developed for 3 h in the presence or absence of 0.1 M sorbitol or 0.1 M KCl. Then cells were subjected to quantification of the amount of DdCAD-1 on the cell surface, cell cohesion assay, and Western blot analysis of amounts of DdCAD-1 in the whole cell lysate and in the membrane fraction. To prepare total cell membranes, NC4 cells were homogenized as described above. The postnuclear supernatant (2 ml) was placed on top of 1 ml of 15% sucrose in GMC buffer, and membranes were pelleted at 33,000 rpm for 1 h at 4°C. Activity of acid phosphatase secreted into the medium was measured as described by Crean and Rossomando (1979).

Alternatively, NC4 cells were developed for 2 h in the presence of 5 and 10  $\mu$ M 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (ICN Biomedical Inc., Aurora, OH), a potent inhibitor of vacuolar H<sup>+</sup>-ATPase (Mellman et al., 1986; Padh et al., 1989). Since NBD-Cl was prepared as a 5 mM stock solution in ethanol, control cells were developed in the presence of 0.2% ethanol. Cells were subjected to quantification of the cell surface-associated DdCAD-1, Western blot analysis, and measurement of acid phosphatase activity secreted into the medium. Morphological observations were carried out using a Zeiss Axiovert 135 inverted microscope.

## Cell Cohesion Assay

Intercellular cohesion was assayed using a modified method (Lam et al., 1981) of the original roller tube assay of Gerisch (1961). After development for a specific period in either hypertonic medium or NBD-Cl, NC4 cells were resuspended in 17 mM phosphate buffer, pH 6.4, at  $3 \times 10^6$  cells per ml. Cell aggregates were dispersed by gentle pipetting. Cells were allowed to re-form aggregates by rotating at 180 rpm on a platform shaker at room temperature. After 20 min, the number of nonaggregated cells, including both singlets and doublets, was scored using a hemocytometer.

## DdCAD-1 Null Mutant

The *cadA* gene encoding DdCAD-1 was disrupted using the restriction enzyme-mediated integration method (Kuspa and Loomis, 1992). A plasmid containing the *cadA* gene (Wong et al., 1996) and the blasticidin S-resistance cassette (Sutoh, 1993) was constructed for DNA integration. DNA transfection was carried out by electroporation, and blasticidin S-resistant transformants were isolated and cloned. The disruption of the *cadA* gene was confirmed by Southern and Western blot analyses.

## In Vitro Reconstitution of DdCAD-1 Import into Contractile Vacuoles

A membrane-free cytosolic fraction prepared from KAX3 cells was used as the source of soluble DdCAD-1 molecules. Cells ( $\sim 2 \times 10^9$  cells) were developed for 6 h in suspension and then lysed at room temperature in 12 ml of 2 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, pH 7.5 (TM buffer) by two passages through a membrane filter with 5- $\mu$ m-diam pores. DTT was added to the postnuclear supernatant at a final concentration of 2 mM and then centrifuged at 40,000 rpm using a Beckman SW40 rotor for 1 h at 4°C. Aliquots of the supernatant were frozen rapidly in liquid nitrogen and stored at -70°C. Protein concentration was determined using the bicinchoninic acid assay kit (Pierce Chemical Co., Rockford, IL) and the protein concentration was adjusted to  $\sim 6$  mg/ml. DdCAD-1 concentration in the cytosol

was 0.2 mg/ml, determined by Western blot analysis using purified DdCAD-1 as a standard.

After 6 h of development in a suspension culture, *cadA*<sup>-</sup> cells ( $5 \times 10^8$ ) were collected and homogenized. The postnuclear supernatant (5 ml) was loaded on a discontinuous sucrose density gradient (28% and 42%, 4 ml each) and centrifuged at 40,000 rpm for 1 h at 4°C. Contractile vacuoles enriched at the interface were collected ( $\sim 300$   $\mu$ l), frozen in aliquots in liquid nitrogen, and stored at -70°C. Protein concentration of this fraction was 2–3 mg/ml. Purified glutathione-S-transferase (GST) protein and anti-GST antibody were prepared as described previously (Zhao and Siu, 1996).

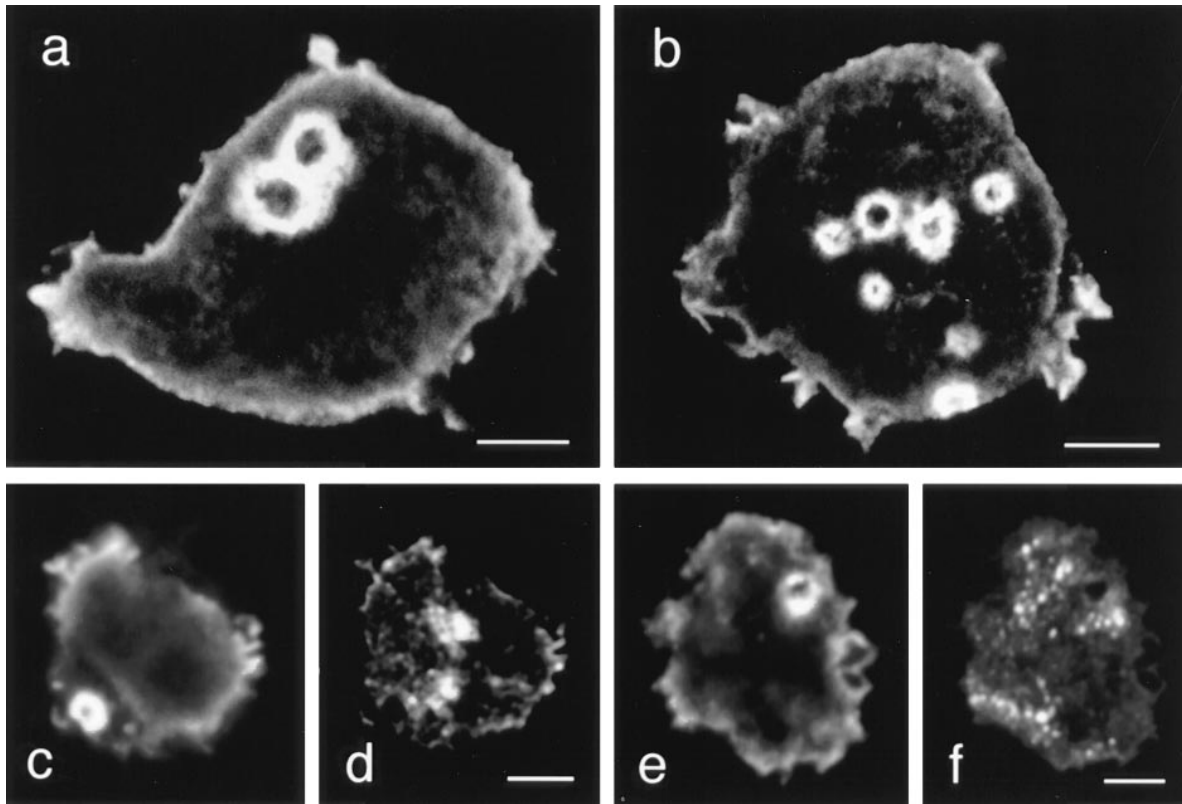
To carry out the in vitro import assay, the cytosolic fraction was clarified by centrifugation at 40,000 rpm for 1 h at 4°C. Then, one-third volume of 60% sucrose in TM buffer was added to the soluble protein fraction. The contractile vacuole fraction was thawed on ice and diluted 1:1 with TM buffer containing 4 mM DTT. 100  $\mu$ l of the cytosol fraction was mixed with 200  $\mu$ l of the contractile vacuole fraction and incubated for 1 h at room temperature. Contractile vacuoles were separated from free proteins by centrifugation through 3 ml of 25% sucrose in TM buffer, on top of a cushion of 0.2 ml of 60% sucrose. Samples were centrifuged at 40,000 rpm using a Beckman SW50.1 rotor for 1 h at 4°C. Contractile vacuoles (500  $\mu$ l) were collected from the 25–60% interface of the sucrose gradient. As a control, purified GST was added to the cytosolic fraction at 0.2 mg/ml. In some experiments, 1 mM ATP and/or an ATP regenerating system containing 40 mM creatine phosphate and 0.2  $\mu$ g/ml creatine kinase (Scott and Klionsky, 1995) were added to the reaction mixture.

## Results

### Immunolocalization of DdCAD-1 in Contractile Vacuoles

DdCAD-1 is a soluble protein that is localized primarily in the cytosol of *Dictyostelium* cells (Brar and Siu, 1993). However, a substantial amount of DdCAD-1 is also present on the cell surface (Sesaki and Siu, 1996). To investigate the mechanism by which DdCAD-1 is transported to the cell surface, immunofluorescence labeling was carried out to determine whether DdCAD-1 was associated with intracellular membrane structures. LSCM revealed that DdCAD-1 was preferentially associated with large cytoplasmic vacuoles, with diameters varying between 1 and 4  $\mu$ m (Fig. 1, *a* and *b*). Usually, more than one DdCAD-1-positive vacuole was present in each cell. DdCAD-1-positive vacuoles appeared as early as after 3 h of development. The morphology and size of the stained vacuoles suggested that they might be contractile vacuoles. To determine whether DdCAD-1 was synthesized on the RER and then targeted to the plasma membrane via the Golgi apparatus, double immunofluorescence labeling was carried out with antibodies directed against DdCAD-1 and gp80. The latter cell adhesion molecule is synthesized and glycosylated using the ER-Golgi pathway (Hohmann et al., 1985, 1987) and was therefore used as a marker for these organelles. The results show that DdCAD-1 did not colocalize with gp80 (Fig. 1, *c* and *d*), suggesting that DdCAD-1 does not associate with either the ER or the Golgi apparatus. Since the *Dictyostelium* lysosome has been shown to be a secretory organelle (Cardelli, 1993; Ruscetti et al., 1994), double immunostaining was carried out to determine whether DdCAD-1 was enriched in lysosomes. Using  $\alpha$ -mannosidase as a marker for lysosomes (Bush and Cardelli, 1989), we found that DdCAD-1 did not colocalize with  $\alpha$ -mannosidase in lysosomes.

Contractile vacuoles are intracellular membrane organelles involved in osmoregulation by excreting water



**Figure 1.** Subcellular localization of DdCAD-1 by immunofluorescence microscopy. KAX3 cells were cultured axenically and then developed for 12 h on coverslips under submerged conditions. Cells were fixed and stained with rabbit antibodies against DdCAD-1 for LSCM (*a* and *b*). Preferential association of DdCAD-1 with large cytoplasmic vacuoles as well as the plasma membrane was observed. Similar results were obtained with cells developed for 6 and 9 h (data not shown). Cells were subjected to double immunostaining using rabbit antibodies against DdCAD-1 (*c* and *e*) and mouse anti-gp80 mAb (*d*) or mouse anti- $\alpha$ -mannosidase mAb (*f*). Bars, 5  $\mu$ m.

into the extracellular medium during transient fusion with the plasma membrane (Zhu and Clarke, 1992; Heuser et al., 1993; Nolte and Steck, 1994). Vacuolar  $H^+$ -ATPase (Fok et al., 1993) and calmodulin (Zhu and Clarke, 1992; Zhu et al., 1993) are known to associate with contractile vacuoles. To demonstrate that DdCAD-1 was indeed associated with contractile vacuoles, double immunofluorescence staining was carried out. Results showed that DdCAD-1 was colocalized to contractile vacuoles with both  $H^+$ -ATPase (Fig. 2, *a* and *b*) and calmodulin (Fig. 2, *c-f*). Serial confocal images through single cells showed that contractile vacuoles were located close to the plasma membrane and vacuoles fused with the plasma membrane were frequently observed (data not shown). Contractile vacuoles fused with the plasma membrane often displayed strong DdCAD-1 and calmodulin staining (Fig. 2, *e* and *f*).

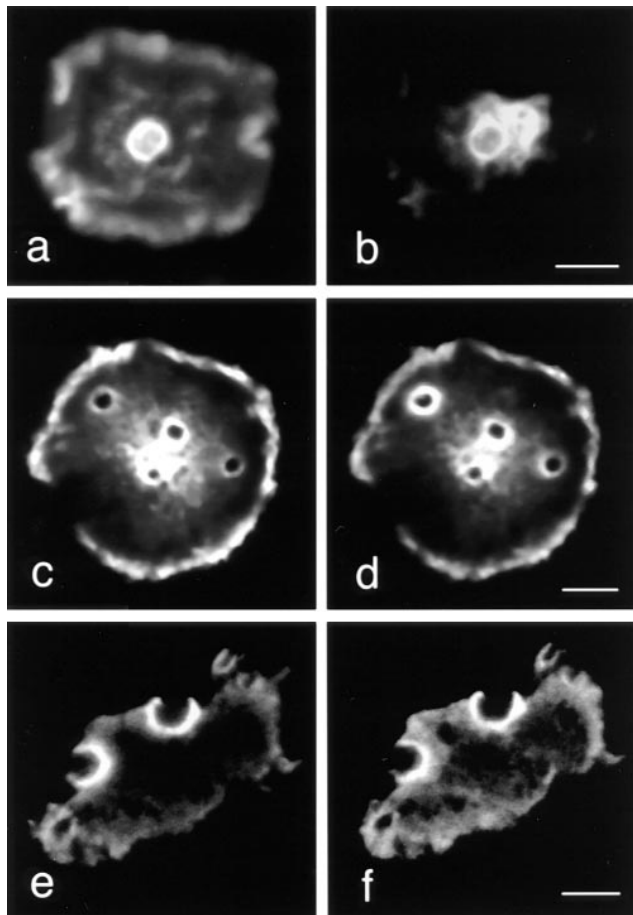
Intravacuolar signals of DdCAD-1 were frequently observed in contractile vacuoles (Fig. 3 *a*). Occasionally, they displayed a punctate staining pattern with regular intervals along the luminal surface of the vacuole (Fig. 3 *b*). In contrast, calmodulin staining was never detected inside contractile vacuoles, suggesting that DdCAD-1 was selectively taken up by contractile vacuoles. Interestingly, strong DdCAD-1 signals were also seen spreading between the lumen of the contractile vacuole and the exterior of the

plasma membrane (Fig. 3, *c* and *d*), implying translocation of DdCAD-1 between these two membranes.

#### *Copurification of DdCAD-1 with Contractile Vacuoles*

The association of DdCAD-1 with contractile vacuoles was confirmed by subcellular fractionation (Fig. 4). Cells were collected at 12 h of development and homogenized. The postnuclear supernatant was fractionated on a continuous sucrose density gradient. Distribution of intracellular organelles, including contractile vacuoles, mitochondria, lysosomes, and plasma membranes, over the gradient were determined by measuring the activity of specific enzyme markers (Fig. 4 *A*) (Padh et al., 1989; Nolte et al., 1991). To determine DdCAD-1 distribution, membranes were pelleted from gradient fractions by ultracentrifugation in the presence of 25 mM NaOH, which was included to remove proteins loosely associated with membranes (Luna et al., 1981), and then analyzed by Western blotting (Fig. 4 *B*).

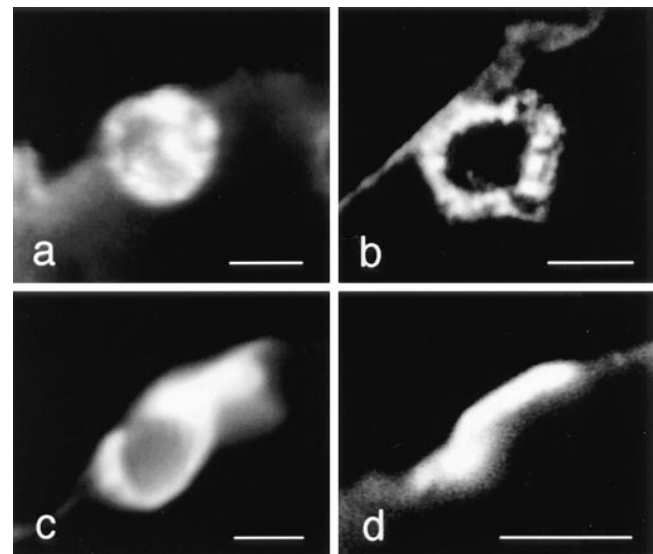
Consistent with our morphological data, DdCAD-1 cofractionated with the peak activity of the contractile vacuole marker enzyme alkaline phosphatase (Nolte and Steck, 1994). In addition to contractile vacuoles, the fractions (15–17) corresponding to the peak of vanadate-sensitive  $H^+$ -ATPase activity, a marker for the plasma membrane,



**Figure 2.** Colocalization of DdCAD-1 with contractile vacuole markers. After development for 12 h under submerged conditions, cells were fixed and then double labeled with rabbit anti-DdCAD-1 antibody (*a*, *c*, and *e*) and mouse anti-calmodulin mAb (*d* and *f*) or mouse anti-H<sup>+</sup>-ATPase mAb (*b*) for LSCM. *e* and *f* show the fusion of contractile vacuoles with the plasma membrane. Bars, 5 μm.

contained a substantial amount of DdCAD-1, indicating its association with the cell membrane. Acid phosphatase released from lysosomes can be found at the top of the sucrose gradient (Nolta et al., 1991). Interestingly, both acid phosphatase and DdCAD-1 were found in fractions 1–3. Since earlier immunostaining results showed that DdCAD-1 was not associated with lysosomes (Fig. 1, *e* and *f*), DdCAD-1 found in the top region of the gradient probably came from the cytosolic pool and not from lysosomes.

To demonstrate that DdCAD-1 was present in the lumen of contractile vacuoles, contractile vacuole preparations were subjected to proteolytic digestion by proteinase K in the presence or absence of 1% Triton X-100 at 37°C for 2 h, and then analyzed by Western blotting using anti-DdCAD-1 and anti-calmodulin antibodies (Fig. 4 C). The level of DdCAD-1 was reduced by <30% when proteolysis was carried out in the absence of detergent. However, DdCAD-1 was digested completely in the presence of Triton X-100. In contrast, calmodulin, which is known to associate with the cytoplasmic surface of contractile vacuoles (Zhu and Clarke, 1992), was completely degraded in the absence of detergent. The protection of DdCAD-1



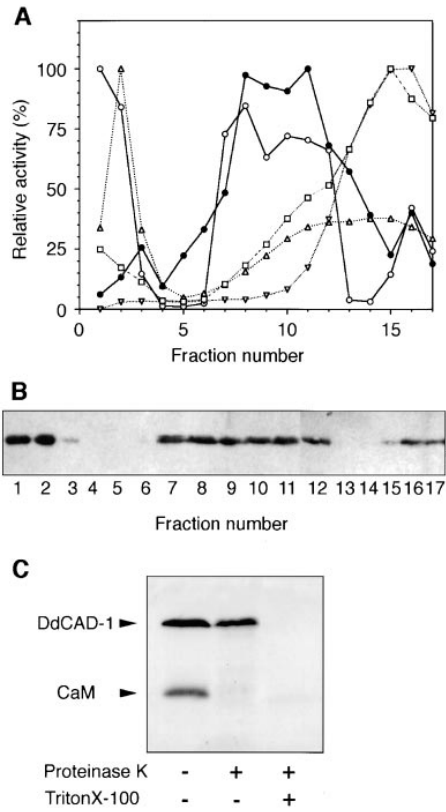
**Figure 3.** Different patterns of DdCAD-1 signal associated with contractile vacuoles. Images taken at a higher magnification frequently revealed intravacuolar signals of DdCAD-1 (*a*). Occasionally, a punctate staining pattern of DdCAD-1 localization on the luminal surface of contractile vacuoles was observed (*b*). Contractile vacuoles were frequently observed in contact with the plasma membrane (*a* and *b*). Upon fusion of contractile vacuoles with the cell membrane, a contiguous staining pattern of DdCAD-1 spanning between the contractile vacuole membrane and the plasma membrane was observed (*c* and *d*). Bars, 3 μm.

from proteolysis by the vacuole membrane indicated the localization of DdCAD-1 in the vacuole lumen.

### ***Inhibition of the Surface Expression of DdCAD-1 by Hypertonic Treatment***

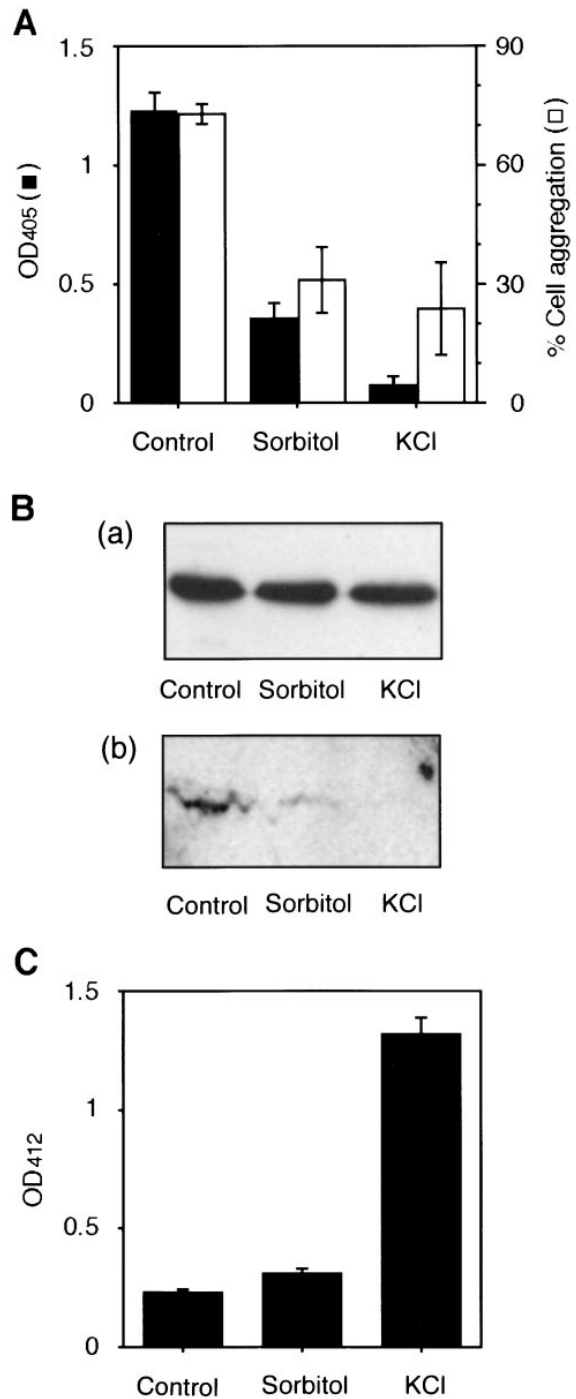
It has been reported that incubation of cells in hypertonic media leads to the collapse of contractile vacuoles (Zhu and Clarke, 1992). If contractile vacuoles represent the major vehicle by which DdCAD-1 is transported to the plasma membrane, hypertonic treatment should inhibit the cell surface expression of DdCAD-1. To test this theory, cells were developed for 3 h in the presence or absence of 0.1 M sorbitol or 0.1 M KCl and the relative level of DdCAD-1 expressed on the cell surface was quantified by ELISA. The levels of surface-associated DdCAD-1 in cells developed in the presence of sorbitol and KCl were ~30 and ~10%, respectively, relative to control cells (Fig. 5 A). Under hypertonic conditions, contractile vacuoles collapsed and DdCAD-1-stained vacuoles were rarely observed (Fig. 6), suggesting that most of the DdCAD-1 failed to enter contractile vacuoles and remained in the cytosol.

Whether a lower level of DdCAD-1 expression on the cell surface would result in the loss of cell–cell adhesion was also investigated. Treated and untreated cells were assayed for cell cohesion in phosphate buffer. Whereas control cells achieved 75% cell aggregation within 20 min, <30% of cells developed in the hypertonic solutions were able to form aggregates (Fig. 5 A). Also, the average size of aggregates was much smaller than those of control cells (data not shown).

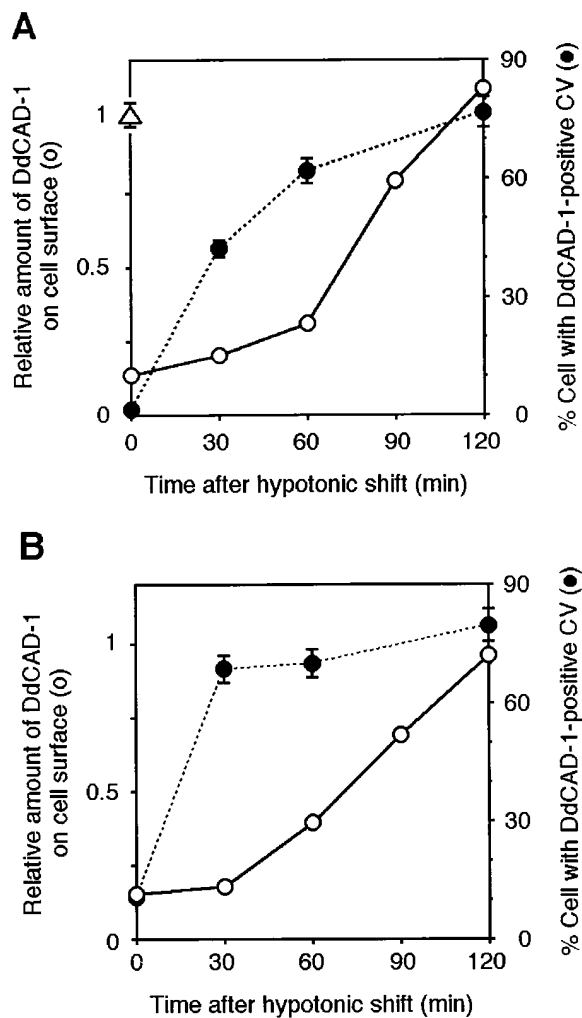


**Figure 4.** Copurification of DdCAD-1 with contractile vacuoles. KAX3 cells were developed for 12 h in liquid culture and then homogenized. The postnuclear supernatant was fractionated on a 30–60% continuous sucrose density gradient by centrifugation at 35,000 rpm for 3 h at 4°C. (A) The distribution of different organelles was determined by assaying specific enzyme markers: alkaline phosphatase for contractile vacuoles (●), vanadate-sensitive H<sup>+</sup>-ATPase for the plasma membrane (□), F<sub>1</sub>F<sub>0</sub>-ATPase for mitochondria (▽), and acid phosphatase for lysosomes (△). (B) Cofractionation of DdCAD-1 with the contractile vacuole marker enzyme was determined by Western blot analysis. A sample of 500 μl was taken from each fraction and diluted with 5 vol of the homogenization buffer. After incubation for 30 min on ice in the presence of 25 mM NaOH, membranes were pelleted, subjected to SDS-PAGE, and then blotted against the anti-DdCAD-1 antibody. The relative amounts of DdCAD-1 in these fractions were estimated by densitometry (○) and shown in A. (C) Presence of DdCAD-1 in the lumen of contractile vacuoles. Proteolytic digestion of DdCAD-1 associated with the contractile vacuole peak fractions in a sucrose density gradient was carried out. A 100-μl sample of the contractile vacuole fraction was incubated with 0.1 mg/ml proteinase K in the presence or absence of 1% Triton X-100 for 2 h at 37°C. After boiling, proteins were separated by SDS-PAGE, followed by Western blot analysis using anti-DdCAD-1 and anti-calmodulin antibodies.

To distinguish between the effects of hypertonic treatment on overall DdCAD-1 expression and its effects on DdCAD-1 transport to the plasma membrane, Western blot analysis was carried out on total cell homogenates and the membrane fraction (Fig. 5 B). No significant difference in the total amount of DdCAD-1 was observed in cells with or without hypertonic treatments. However, the level of DdCAD-1 in the membrane fraction was much reduced in cells treated with either sorbitol or KCl. There-



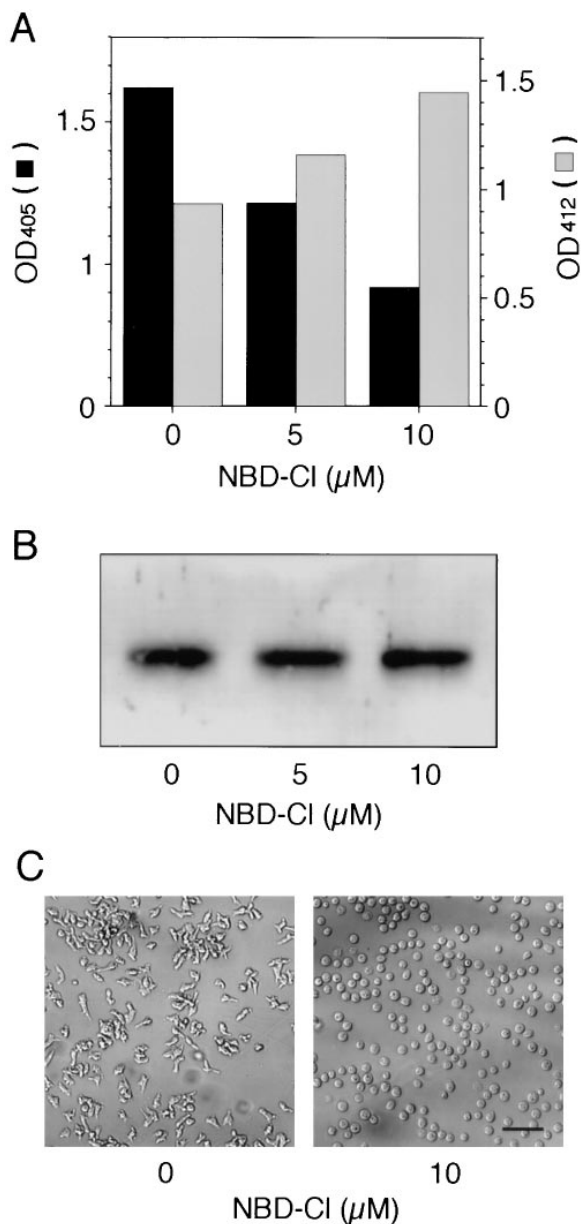
**Figure 5.** Inhibition of DdCAD-1 transport and cell–cell adhesion by hypertonic conditions. NC4 cells were collected and developed for 3 h in the presence of 0.1 M sorbitol or 0.1 M KCl. (A) The relative amounts of DdCAD-1 expressed on the cell surface of the treated and untreated cells were determined by ELISA (solid bars). Background was subtracted using vegetative cells that do not contain DdCAD-1 (Knecht et al., 1987). Effects of hypertonic treatment on cell–cell adhesion were determined using the cell reassociation assay (see Materials and Methods) (open bars). Values represent the mean ± SD (*n* = 3). (B) Total cellular DdCAD-1 (a) and DdCAD-1 associated with the membrane fraction (b) were examined by Western blot analysis. (C) The activity of acid phosphatase secreted into the medium was determined as described (Crean and Rossomando, 1979).



**Figure 6.** Time course of DdCAD-1 transport after hypotonic shift. KAX3 cells were developed for 4, 4.5, 5, 5.5, and 6 h in either 0.1 M sorbitol (*A*) or 0.1 M KCl (*B*) and then transferred to 17 mM phosphate buffer, pH 6.4. All cell samples were collected at 6 h, fixed, and stained with anti-DdCAD-1 antibody. The number of cells with DdCAD-1-positive contractile vacuoles was scored (●). The percentage of positive control cells (Δ) is shown along the y-axis of *a*. Values represent the mean of three experiments; 100 cells were scored in each experiment. The amounts of DdCAD-1 present on the cell surface were determined by ELISA, and values were normalized to that of control cells (○).

fore, the data indicate that hypertonic conditions affect the transport of DdCAD-1 to the cell surface and not its accumulation in the cytosol. It is therefore evident that the surface expression of DdCAD-1 is dependent on contractile vacuoles.

Lysosomes have been shown to secrete lysosomal enzymes during development (Cardelli, 1993). Our earlier data showed that the lysosomal enzyme acid phosphatase and DdCAD-1 cofractionated in the top fractions of the sucrose gradient. To assess the possibility that DdCAD-1 might be transported via lysosomes, we examined the secretion of acid phosphatase into the outer media under hypertonic conditions. If DdCAD-1 transport makes use of the lysosomal secretory pathway, one would predict the in-



**Figure 7.** Inhibition of DdCAD-1 transport by NBD-Cl. NC4 cells were collected and developed for 2 h in the presence of NBD-Cl. (*A*) The relative amounts of DdCAD-1 expressed on the cell surface of the treated and untreated cells were determined by ELISA (black bars). Background was subtracted using vegetative cells that do not contain DdCAD-1. The activity of secreted acid phosphatase in the outer media (gray bars) was determined as described previously (Crean and Rossomando, 1979). (*B*) Total cellular DdCAD-1 was examined by Western blot analysis. (*C*) Morphologies of cells were observed using conventional phase contrast microscopy. Bar, 50 μm.

hibition of lysosomal enzyme secretion by hypertonic treatments. In contrast with DdCAD-1, the secretion of acid phosphatase was stimulated by 1.3- and 5-fold in the presence of 0.1 M sorbitol and 0.1 M KCl, respectively. It is therefore unlikely that lysosomes are involved in the transport of DdCAD-1.

### Induction of Surface Expression of DdCAD-1 by Hypotonic Shift

The reverse shift of environmental osmolarity from a higher strength to a lower one induces reappearance of contractile vacuoles (Zhu and Clarke, 1992). It was therefore of interest to examine whether the surface expression of DdCAD-1 was resumed after a hypoosmotic shift. After development for various time intervals in the presence of sorbitol or KCl, cells were transferred to 17 mM phosphate buffer and all cell samples were collected at 6 h for assays. The appearance of DdCAD-1-positive contractile vacuoles was monitored by fluorescence microscopy, and the accumulation of DdCAD-1 on the cell surface was determined using ELISA (Fig. 6). Initially, only 5–10% of the cells contained DdCAD-1-positive contractile vacuoles. Most cells contained one or more DdCAD-1-positive contractile vacuoles by 60 min after the shift. The inhibition of DdCAD-1 expression on the cell surface by hypertonic medium was reversible, and the accumulation of DdCAD-1 on the cell surface followed the appearance of DdCAD-1-positive contractile vacuoles. DdCAD-1 accumulated rapidly on the cell surface after an initial delay of 30–60 min. The surface level of DdCAD-1 became indistinguishable from that of control cells after 2 h. The temporal relationships between the appearance of contractile vacuoles and the surface accumulation of DdCAD-1 are consistent with the notion that DdCAD-1 is transported to the plasma membrane via contractile vacuoles.

### Inhibition of the Surface Expression of DdCAD-1 by NBD-Cl

Since it has been reported that NBD-Cl is a specific inhibitor to vacuolar-type H<sup>+</sup>-ATPase (Mellman et al., 1986; Padh et al., 1989), we used this reagent to inhibit contractile vacuole activity and its effects on the expression of DdCAD-1 on the cell surface. Cells were developed for 2 h in the presence or absence of 5 and 10 μM NBD-Cl. Treated cells lost their contractile vacuole function and displayed a round and swollen morphology, whereas control cells spread well and adopted a more elongated shape (Fig. 7 C). Although treated cells appeared to be swollen, most cells remained intact during the 2-h incubation period in 17 mM phosphate buffer. Cell lysis began to occur at 20 μM NBD-Cl.

The levels of surface-associated DdCAD-1, in cells de-

veloped in the presence of 5 and 10 μM NBD-Cl, were quantified. NBD-Cl-treated cells showed significant reductions in their levels of DdCAD-1 on the cell surface (Fig. 7 A). In the presence of 10 μM NBD-Cl, the cell surface level of DdCAD-1 dropped by 65%. However, no significant difference in the total cellular level of DdCAD-1 was observed in cells with or without NBD-Cl treatments (Fig. 7 B). When the effects of NBD-Cl on the secretion of the lysosomal enzyme acid phosphatase were examined, the amount of secreted acid phosphatase increased 1.5-fold in 10 μM NBD-Cl (Fig. 7 A). These results indicate that NBD-Cl inhibited contractile vacuole activity and the transport of DdCAD-1, but it had no effect on DdCAD-1 accumulation and the lysosomal secretion pathway.

### In Vitro Reconstitution of DdCAD-1 Import into Contractile Vacuoles

To test whether isolated contractile vacuoles could selectively import DdCAD-1, a cell-free reconstitution assay was developed using a mutant strain with the *cadA* gene, which encodes DdCAD-1, disrupted by the blasticidin-resistant cassette. The *cadA*<sup>-</sup> mutant cells did not express DdCAD-1 but contained normal contractile vacuoles. Immunofluorescence microscopy showed the presence of calmodulin-positive contractile vacuoles inside these mutant cells (Fig. 8 A), and this was further demonstrated by Western blot analysis of contractile vacuoles purified on a sucrose density gradient (Fig. 8 B).

The reconstitution assay was carried out as illustrated schematically in Fig. 9 A. Contractile vacuoles were isolated from the *cadA*<sup>-</sup> cells and then mixed with the soluble protein fraction obtained from wild-type cells. After 1 h of incubation at room temperature, the contractile vacuoles were repurified using a discontinuous sucrose density gradient. The uptake of DdCAD-1 by contractile vacuoles was examined by proteinase K (10 μg/ml) digestion of the repurified vacuoles followed by Western blot analysis (Fig. 9 B). If DdCAD-1 was imported from the wild-type cytosol into the contractile vacuoles of *cadA*<sup>-</sup> cells during incubation, DdCAD-1 should have become resistant to proteolytic digestion in the absence of detergent. Indeed, the DdCAD-1 associated with contractile vacuoles was resistant to proteinase K digestion, indicating the translocation of DdCAD-1 into the lumen of these vacuoles. In contrast, contractile vacuole-associated calmodulin was degraded

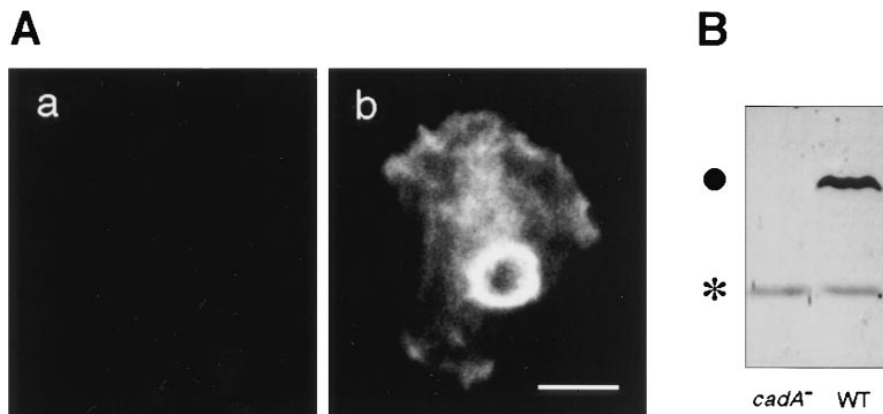
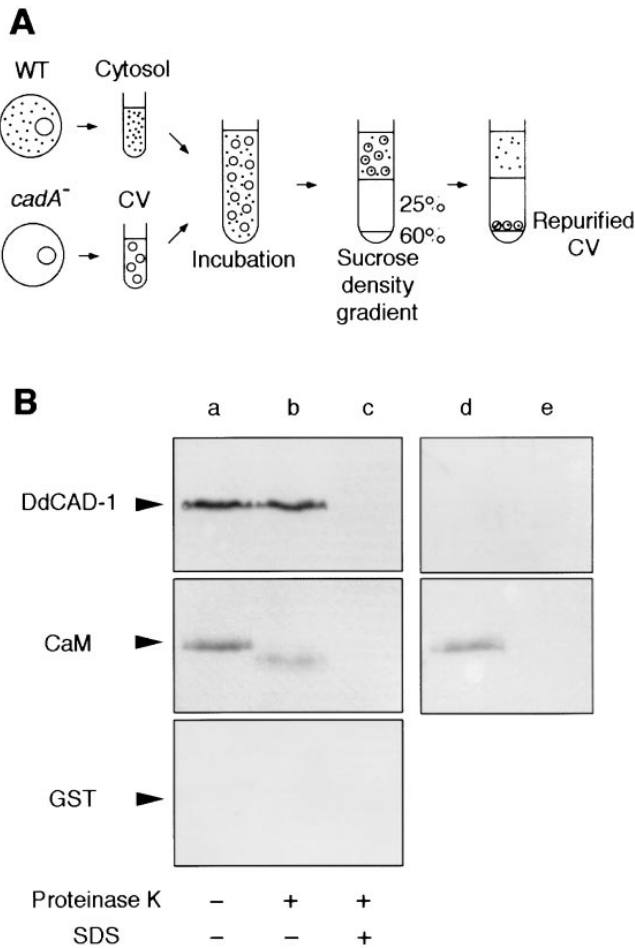


Figure 8. *cadA*<sup>-</sup> cells contained contractile vacuoles devoid of DdCAD-1. (A) The DdCAD-1 import assay made use of *cadA*<sup>-</sup> cells that contained contractile vacuoles devoid of DdCAD-1. The *cadA*<sup>-</sup> cells were double immunostained with anti-DdCAD-1 (a) and anti-calmodulin (b) antibodies. (B) A Western blot of the contractile vacuole-enriched fractions isolated from mutant and wild-type (WT) cells was stained with both anti-DdCAD-1 antibody (●) and anti-calmodulin antibody (\*).





**Figure 9.** In vitro import of DdCAD-1 into contractile vacuoles. (A) Schematic drawing of the experimental protocol. The membrane-depleted cytosol fraction and the contractile vacuole-enriched fraction were prepared from KAX3 cells and *cadA*<sup>-</sup> cells, respectively. These fractions were mixed at a ratio of 2:1 and incubated for 1 h at room temperature. As a negative control, GST was added to the assay mixture at 0.2 mg/ml. Then contractile vacuoles were reisolated by centrifugation through a discontinuous sucrose density gradient and subjected to Western blot analysis. (B) Western blots showing the incorporation of DdCAD-1 into contractile vacuoles (lanes a–c). After incubation with the cytosol fraction derived from wild-type cells, repurified contractile vacuoles were subjected to proteinase K (10 μg/ml) digestion in the presence or absence of SDS. Additional controls included the incubation of the *cadA*<sup>-</sup> contractile vacuoles with buffer alone (lane d) and the incubation of wild-type cytosol with buffer alone (lane e).

under the same condition, yielding a partially digested fragment (Fig. 9 B). Addition of either SDS or Triton X-100 resulted in the complete digestion of DdCAD-1.

Several controls were carried out to demonstrate that the import of DdCAD-1 into contractile vacuoles was a selective process. First, when the wild-type cytosolic fraction was incubated with buffer, no detectable amount of DdCAD-1 was recovered from the interface region of the sucrose gradient, demonstrating that the cytosolic fraction was devoid of wild-type contractile vacuoles (Fig. 9 B, lane e). Second, when GST, a soluble protein with a molecular size

similar to DdCAD-1, was added to the cytosol, no GST was found associated with or imported into contractile vacuoles (Fig. 9 B). Also, calmodulin, which is present both in the cytosol and on the vacuole surface, did not enter the vacuole lumen. Finally, when the contractile vacuole membrane was disrupted by low osmotic shock before the import reaction, DdCAD-1 was degraded by proteinase K in the absence of SDS (data not shown). Taken together, these results ruled out the presence of contaminating wild-type contractile vacuoles or nonspecific uptake of soluble DdCAD-1 by damaged contractile vacuoles.

Since the contractile vacuole fraction used in the DdCAD-1 import assay was contaminated by plasma membrane and lysosomes, experiments were carried out to determine whether plasma membrane vesicles and lysosomes also contributed to DdCAD-1 uptake. A series of discontinuous sucrose density gradients were performed to obtain membrane fractions that contained various amounts of contractile vacuoles, plasma membrane, and lysosomes, and the import of DdCAD-1 into protease-resistant compartments in these fractions was assessed. As shown in Table I, the DdCAD-1 import activity was not affected by the increase or decrease in the relative amounts of plasma membrane and lysosomes. In contrast, the DdCAD-1 import activity was dependent on the amount of contractile vacuoles in these fractions. When the relationship between contractile vacuoles and DdCAD-1 import was subjected to linear regression analysis, a strong positive correlation was observed between DdCAD-1 import activity and the activity of the contractile vacuole marker alkaline phosphatase, with a correlative coefficient of +0.997. Neither the vanadate-sensitive H<sup>+</sup>-ATPase activity nor the acid phosphatase activity showed positive correlation with DdCAD-1 import.

The import of DdCAD-1 was enhanced by ATP and the ATP regeneration system. When 1 mM ATP and the ATP regeneration system were added to the import mixture, a twofold increase in DdCAD-1 uptake by contractile vacuoles was observed (Fig. 10 A). However, when only ATP was added, DdCAD-1 import was indistinguishable from the control. Similarly, the regeneration system itself did not have any stimulatory effect (data not shown). To exclude the possibility that ATP and the regeneration system increased the yield of the contractile vacuole membrane, we measured alkaline phosphatase activity in the repurified contractile vacuole membrane fractions and no significant difference was observed (Fig. 10 B). Also, the association of calmodulin with contractile vacuoles was not affected by the inclusion of ATP and the ATP regeneration system (Fig. 10 A).

## Discussion

In this paper we have provided evidence for a novel protein transport pathway involved in the presentation of the cell adhesion molecule DdCAD-1 on the surface of *Dictyostelium* cells. DdCAD-1 is present primarily in the cytoplasm of the cell (Brar and Siu, 1993), and it begins to accumulate on the cell surface in the preaggregation stage of development (Sesaki and Siu, 1996). DdCAD-1 lacks a hydrophobic signal peptide sequence and is not associated with the Golgi apparatus or the ER, suggesting that it is

Table I. Distributions of Organelle Markers and DdCAD-1 Import Activity in Different Membrane Fractions

	Activity in the interface fraction			
	30/42%	30/38%	34/60%	38/60%
Alkaline phosphatase	77.9 ± 1.1 (1)	51.1 ± 1.7 (0.66)	29.5 ± 0.37 (0.38)	20.3 ± 0.37 (0.38)
Vi-sensitive H <sup>+</sup> -ATPase	31.0 ± 6.4 (1)	30.0 ± 5.1 (0.97)	83.3 ± 8.7 (2.7)	44.4 ± 8.1 (1.4)
Acid phosphatase	16.0 ± 2.5 (1)	6.7 ± 0.8 (0.42)	84.1 ± 5.3 (5.3)	83.0 ± 6.0 (5.2)
DdCAD-1 import	474812 (1)	329045 (0.69)	208917 (0.44)	129624 (0.27)

After development for 6 h in 17 mM phosphate buffer, pH 6.5, *cadA*<sup>-</sup> cells were homogenized, and a postnuclear supernatant (1 ml) was separated on four discontinuous sucrose gradients (30 and 42%, 30 and 38%, 34 and 60%, and 38 and 60%) at 40,000 rpm for 1 h. 300- $\mu$ l fractions were collected from the interface. The activity of marker enzymes and the DdCAD-1 import activity were measured as described in Materials and Methods. To determine the total activity of each marker enzyme, membranes in a postnuclear supernatant (1 ml) were pelleted at 18,000 rpm for 30 min, resuspended in 300  $\mu$ l of 10 mM Tris-HCl, pH 7.5, and 2 mM MgCl<sub>2</sub>, and used for enzyme assay. To measure the DdCAD-1 import activity, 100  $\mu$ l of each fraction was used. All enzyme activities were expressed relative to the total cellular activity (100%). The relative amounts of DdCAD-1 taken up by membrane vesicles were determined by densitometric tracing of Western blots. Ratios relative to the activity in the 30/42% interface are shown in parentheses. The DdCAD-1 import activity was positively correlated with alkaline phosphatase activity with a correlation coefficient of 0.997, but not with Vi-sensitive H<sup>+</sup>-ATPase activity (-0.526) or acid phosphatase activity (-0.848).

not transported to the plasma membrane via the classical pathway. Our morphological observation indicates that DdCAD-1 is concentrated in contractile vacuoles. Biochemical analysis of purified contractile vacuoles confirms the presence of a substantial amount of DdCAD-1 inside contractile vacuoles. Hypertonic treatment of cells leads to the collapse of contractile vacuoles and inhibits the accumulation of DdCAD-1 on the cell surface. However, hypotonic shift results in the rapid reappearance of DdCAD-1-positive contractile vacuoles and the accumulation of DdCAD-1 on the cell surface. Furthermore, NBD-Cl, a specific inhibitor of vacuolar H<sup>+</sup>-ATPase, represses the activity of contractile vacuoles and inhibits the accumulation of DdCAD-1 on the cell surface. Taken together, these results indicate that contractile vacuoles represent the major vehicle by which DdCAD-1 is transported from the cytoplasm to the plasma membrane.

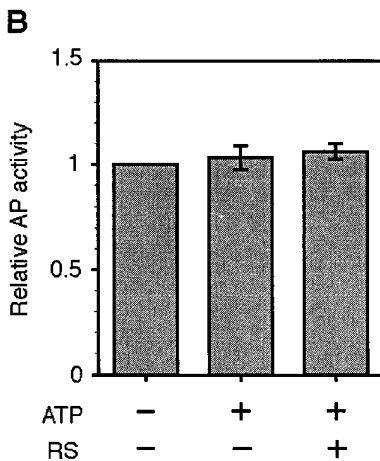
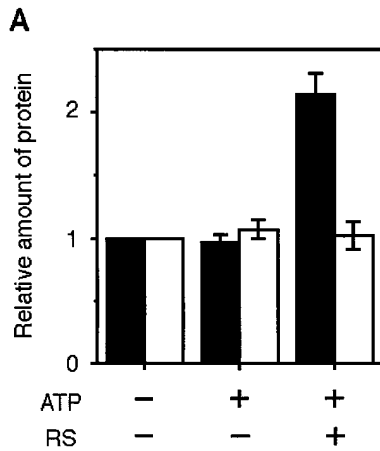
Contractile vacuoles are intracellular organelles found in most freshwater protozoa that are responsible for osmoregulation in cells. Both appearance and behavior of contractile vacuoles are tightly correlated with environmental osmolarity (Patterson, 1980; Zeuthen, 1992). Light microscopy shows that contractile vacuoles repeat to fill and empty, and such repetitive behavior is accelerated when cells are placed in hypotonic media, suggesting that contractile vacuoles function by excreting excess water from the cytosol. On the other hand, placing cells in hypertonic media leads to the collapse of contractile vacuoles. Furthermore, when a dominant-negative form of rabD, a small GTPase localized on contractile vacuoles, is overexpressed in *Dictyostelium* cells, the contractile vacuoles exhibit abnormal morphology and fail to respond to environmental osmolarity, resulting in a swollen cell shape in hypotonic media (Bush et al., 1996). In addition to this classical view of contractile vacuole as a water-excretory organelle, our present data define a new role for contractile vacuoles in protein transport.

Although the lysosome has been shown to be a secretory organelle, capable of releasing lysosomal enzymes into the medium during growth and development in *Dictyostelium* (Cardelli, 1993), several lines of evidence rule out the involvement of lysosomes in DdCAD-1 transport. First, immunofluorescence microscopy showed that DdCAD-1 was not enriched in lysosomes. Second, hypertonic conditions stimulated secretion of the lysosomal enzyme acid phosphatase, whereas DdCAD-1 transport to the cell sur-

face was inhibited. Third, NBD-Cl also exerted opposite effects on DdCAD-1 transport and lysosomal secretion. Fourth, fractions enriched in lysosomes did not show a corresponding increase in the DdCAD-1 import activity. Finally, it has been shown that all of the lysosomal enzymes examined so far contain classical signal peptides at their NH<sub>2</sub> termini (Cardelli, 1993). However, DdCAD-1 does not contain a signal peptide (Wong et al., 1996).

A growing number of soluble proteins that lack an NH<sub>2</sub>-terminal signal peptide are known to be transported to the cell surface or secreted into the medium via nonclassical pathways. Examples of these proteins include galectin-1 (Cooper and Barondes, 1990; Cleves et al., 1996),  $\alpha$ -factor (Kuchel et al., 1989), interleukin-1 $\beta$  (Rubartelli et al., 1990; Siders and Mizel, 1995), thioredoxin (Rubartelli et al., 1992), and basic FGF (Florkiewicz et al., 1995). Previous studies have identified several protein transporters that are involved in the secretion of these proteins, including several members of the ATP-binding cassette superfamily localized in the plasma membrane (for reviews see Higgins, 1992; Fath and Kolter, 1993). The use of these transporters in DdCAD-1 transport cannot be ruled out. However, our results indicate that, even though a plasma membrane-associated DdCAD-1 transport mechanism does exist, its contribution to DdCAD-1 transport is probably minimal (see Table I). The major DdCAD-1 transport pathway appears to depend on contractile vacuoles.

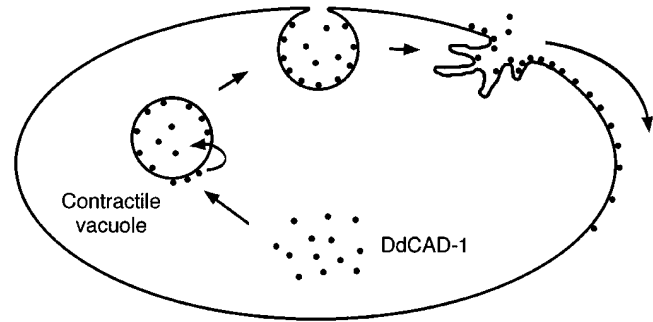
Many steps along the classical transport pathway, including targeting of precursor protein to the ER membrane (Andrews et al., 1989), protein translocation across the membrane (Hansen et al., 1986), vesicle-mediated intercompartmental protein transport (Balch et al., 1984; Baker et al., 1988), and fusion of secretory vesicles with the plasma membrane (Crabb and Jackson, 1985), have been reconstituted successfully in vitro and studied extensively (Pryer et al., 1992; Rothman, 1994; Rapoport et al., 1996). However, nonclassical protein transport pathways are much less well characterized. We propose that the contractile vacuole-mediated protein transport pathway may involve a minimum of four steps, as illustrated schematically in Fig. 11. Initially, DdCAD-1 is recognized by contractile vacuoles and binds to specific components on the cytoplasmic surface of contractile vacuoles. The binding of DdCAD-1 on the contractile vacuole membrane will lead to the translocation of DdCAD-1 across the contractile vacuole membrane. This is followed by the association of



**Figure 10.** Enhancement of DdCAD-1 import into contractile vacuoles by a combination of ATP and an ATP regeneration system. (A) The import assay was carried out in the presence of ATP and an ATP regeneration system (RS), and the relative amounts of DdCAD-1 (solid bars) and calmodulin (open bars) recovered in the contractile vacuoles fraction were quantified by densitometry. (B) The contractile vacuole fractions were solubilized in 0.2% Triton X-100, and the relative amounts of alkaline phosphatase (AP) activity were determined. Values represent the mean  $\pm$  SD ( $n = 3$ ).

DdCAD-1 with an “anchoring” protein on the luminal surface of contractile vacuoles. We envision that one end of the DdCAD-1 molecule is associated with an integral membrane protein, whereas the cell adhesion activity is associated with another segment of the molecule. As the contractile vacuole fuses with the plasma membrane, the anchored DdCAD-1 molecules move laterally from the vacuole membrane to the plasma membrane.

As a first step to characterize this pathway biochemically, we have established an *in vitro* reconstitution system and have successfully demonstrated the import of DdCAD-1 using isolated contractile vacuoles and cytosolic proteins. Our results clearly showed that DdCAD-1 in the wild-type cytosol was taken up by contractile vacuoles isolated from *cadA*<sup>-</sup> cells, whereas GST and calmodulin failed to enter the contractile vacuole. The import of DdCAD-1 is therefore a highly selective process, which probably involves a specific recognition mechanism similar to those found in



**Figure 11.** Schematic drawing showing contractile vacuole-mediated transport of DdCAD-1 from the cytosol to the plasma membrane.

other organelle import systems (Schatz and Dobberstein, 1996). The initial binding of DdCAD-1 on contractile vacuoles can be accomplished by a specific signal sequence on DdCAD-1 or by the formation of a complex of DdCAD-1 and other cytosolic proteins, which in turn is recognized by specific receptors on the contractile vacuole membrane. The import mechanism is enhanced by exogenously added ATP and an ATP regeneration system, suggesting the involvement of specific ATP-dependent transporter(s) in the contractile vacuole membrane. This cell-free system should facilitate a biochemical approach in our future characterization of the early steps involved in this protein transport pathway and allow the identification of membrane components involved in DdCAD-1 translocation.

The fusion of contractile vacuoles with the plasma membrane and their subsequent contraction have been studied fairly extensively. When contractile vacuoles become filled with water, the contents are discharged into the medium through the pore formed by fusion between the contractile vacuole and the plasma membrane (Heuser et al., 1993). It has been proposed that forces involved in pushing away the contents are generated by contraction of the contractile vacuole membrane driven by the actin microfilament cytoskeleton covering the cytoplasmic surface of contractile vacuoles (Baines et al., 1995). Myosin I apparently plays a crucial role in the contraction process, since mechanical loading of anti-myosin I antibodies into *Acanthamoeba* cells leads to cell burst in a hypotonic solution (Doberstein et al., 1993).  $\alpha$ -Actinin is known to associate with contractile vacuoles (Furukawa and Fehheimer, 1994), suggesting that it may also have a role in vacuole contraction.

The accumulation of DdCAD-1 on the cell surface may result from the lateral movement of DdCAD-1 from the luminal surface of contractile vacuoles to the plasma membrane after fusion of these two membranes. Since cells are also known to release soluble DdCAD-1 into the medium during development (Siu et al., 1997), it is possible that some of these molecules will bind back to the cell surface by their association with the DdCAD-1 anchoring protein. We have obtained preliminary evidence for the involvement of an integral membrane protein that can anchor soluble DdCAD-1 to the plasma membrane (Brar, 1994). Whether the same protein is involved in the anchoring of soluble DdCAD-1 on the luminal side of the contractile vacuole membrane remains to be determined.

DdCAD-1 is expressed soon after the initiation of development. However, it is present on the cell surface only for a discrete period of time, mediating cell-cell adhesion in the preaggregation stage (Sesaki and Siu, 1996). Prevention of cell-cell adhesion using anti-DdCAD-1 Fab in the early stages of development blocks the cAMP-mediated stimulation of gp80 expression at the aggregation stage, suggesting that cell-cell adhesion mediated by DdCAD-1 may be involved in signaling pathways that regulate gene expression (Desbarats et al., 1994). Interestingly, upon the formation of stable cell-cell contacts, surface-associated DdCAD-1 leaves the plasma membrane and becomes internalized (Sesaki and Siu, 1996). The observation that DdCAD-1 is enriched in endosomes suggests that internalization of DdCAD-1 may be mediated by an endocytic pathway (Adessi et al., 1995). The presentation and subsequent removal of DdCAD-1 thus represent a dynamic process, which may be used to initiate and fine tune the adhesive interactions among cells during development.

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