The outer dynein arm-docking complex (ODA-DC) targets the outer dynein arm to its correct binding site on the flagellar axoneme. The *Chlamydomonas* ODA-DC contains three proteins; loss of any one prevents normal assembly of the outer arm, leading to a slow, jerky swimming phenotype. We showed previously that the smallest ODA-DC subunit, DC3, has four EF-hands (Casey, D. M., Inaba, K., Fazour, G. J., Takada, S., Wakabayashi, K., Wilkerson, C. G., Kamiya, R., and Witman, G. B. (2005) *Mol. Biol. Cell* 14, 3650–3663). Two of the EF-hands fit the consensus pattern for calcium binding, and one of these contains two cysteine residues within its binding loop. To determine whether the predicted EF-hands are functional, we purified bacterially expressed wild-type DC3 and analyzed its calcium-binding potential in the presence and absence of dithiothreitol and Mg2+. The protein bound one calcium ion with an affinity (K_d) of ~1 × 10^{-8} M. Calcium binding was observed only in the presence of dithiothreitol and thus is redox-sensitive. DC3 also bound Mg2+ at physiological concentrations but with a much lower affinity. Changing the essential glutamate to glutamine in both EF-hands eliminated the calcium binding activity of the bacterially expressed protein. To investigate the role of the EF-hands in vivo, we transformed the modified DC3 gene into a *Chlamydomonas* insertional mutant lacking DC3. The transformed strain swam normally, assembled a normal number of outer arms, and had a normal photoshock response, indicating that the Glu to Gln mutations did not affect ODA-DC assembly, outer arm assembly, or Ca2+-mediated outer arm activity. Thus, DC3 is a true calcium-binding protein, but the function of this activity remains unknown.

Intracellular calcium plays an important role in the control of eukaryotic cilia and flagella. For example, calcium signals control the beat frequency of mammalian airway cilia (1), the hyperactivation of mammalian sperm flagella (2), the reversal of ciliary beating in ciliates (3), and phototaxis and photoshock in the biflagellate alga *Chlamydomonas reinhardtii* (for review see Ref. 4). Despite the important role that calcium plays in these processes, little is known about the biochemical pathways or the calcium-binding proteins involved. A few Ca2+-binding proteins, e.g. calmodulin (5, 6), centrin/caltractin (7), and dynein light chain 4 (see Ref. 8 and see below), have been identified within the axoneme, but their precise functions in Ca2+-mediated flagellar behavior have not yet been clearly defined. Given the complexity of the axoneme and the likely need to modulate the activity of multiple axonemal structures to achieve a coordinated change in waveform in response to changes in Ca2+ concentration, it is probable that the axoneme contains additional Ca2+-binding proteins. Identification and characterization of these proteins will be essential for understanding the molecular basis for the Ca2+ control of ciliary and flagellar waveform.

*Chlamydomonas* normally swims forward by means of a “breaststroke”-like beat pattern in which the flagella propagate asymmetrical bends. The photoshock response, which is induced by a strong step-up light stimulus and mediated by an increase in intraflagellar Ca2+ from ~10^{-6} to ~10^{-5}–10^{-4} M, consists of an abrupt cessation of forward swimming, followed by a brief period of backward swimming during which the flagella propagate symmetrical bends, and finally a return to the normal asymmetrical bending pattern and forward swimming (4). The generation of symmetrical bends during photoshock requires the outer dynein arms (9, 10), suggesting that the outer arms are regulated by Ca2+. One outer dynein arm light chain, LC4, is a Ca2+-binding protein, and it has been hypothesized that this subunit may have a role in mediating outer arm function during photoshock (8); however, there currently is no experimental evidence in support of such a role for LC4.

In the axoneme, the outer arms are in direct contact with the outer dynein arm-docking complex (ODA-DC) (11), which targets the outer arm to its correct binding site on the doublet microtubule (12). The ODA-DC contains three subunits, termed DC1, DC2, and DC3, with masses of 83, 62, and 21 kDa, respectively. DC1 and DC2 are predicted to be long coiled-coil proteins that have a structural role in assembling the ODA-DC, and loss of either of these results in complete loss of the outer arm function during photoshock (8); however, there currently is no experimental evidence in support of such a role for DC4.

1 The abbreviations used are: LC4, the 18-kDa calcium-binding outer dynein arm light chain; ODA-DC, outer dynein arm-docking complex; ARG7, the argininosuccinate lyase gene of *Chlamydomonas*; CaM, calmodulin; CTER, a congruent group of EF-hand proteins including calmodulin, troponin C, and the essential and regulatory light chains of myosin; DTT, dithiothreitol; GST, glutathione S-transferase; TnC, troponin C; TnI, troponin I.

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EXPERIMENTAL PROCEDURES

**Strains**—C. reinhardtii strains used in this study include the following: g1 (nit1, mt+)(15); H8– (arg7, mt–) (obtained from Dr. P. Lefebvre, University of Minnesota); V06 (oda14::Nit1, nit1, mt+)(13), generated by insertional mutagenesis of g1; 026.2 (oda14-1::Nit1, arg7, mt+); offspring of V06 × H8– (cross); and transformants d58, d65, d76, e20, e62, and e84 obtained by transforming strain 026.2 with a wild-type (transformants d58, d65, and d76) or a modified (transformants e20, e62, and e84) ODA14 genomic clone and pARG7.8(16).

**Growth Media**—The following media were used: M medium (17) modified to contain 0.0022 M KH2PO4 and 0.00171 M K2HPO4 and supplemented with 0.0075 M L-glutamine, 0.0075 M adenine (present in this study); R medium supplemented with 50 μg/ml arginine; and TAP (18).

**Site-directed Mutagenesis**—Starting with the plasmid clone pDC3S-2 (14), which contains the intronless wild-type DC3 (ODA14) gene, two sequential rounds of mutagenesis (Chameleon Site-directed Mutagenesis Kit, Stratagene, La Jolla, CA) were used to create point mutations in the DC3 gene. One primer (E74Q, 5'-CTGCATCAGCCT-TCTCAATTCAGCAGCTATAC-3') was designed to inactivate the first consensus EF-hand by changing the highly conserved glutamate residue in position 12 of the calcium-binding loop to a glutamine. After sequencing (DNA Sequencing Facility, Iowa State University, Ames) to confirm that the first consensus EF-hand had been mutated, a second primer (E152Q, 5'-CCTCACCTGTCGAGCTTTCTGACCTGCATTCGCG-3') was used to make a similar Glu to Gln mutation in position 12 of the calcium-binding loop to glutamine. After sequencing (DNA Sequencing Facility, Iowa State University, Ames) to confirm that the second mutation was correct, the construct was named pDC3Q2-9.

**In Vitro Expression of Wild-type and Mutant DC3**—The DC3 coding region of either pDC3S-2 or pDC3Q2-9 was amplified by the PCR using the primers DCFPF-1 (5'-CCGGGATCCATGGCGAGTGCC-3') and DCFPR-1 (5'-CCGGATCTCCATGTGGCTTCTGCG-3'). Amplification with these primers resulted in a 5' BamHI site directly upstream of the DC3 start codon and a 3' BamHI site directly downstream of the stop codon. PCR products were purified using the QiAquick PCR Purification Kit (Qiagen, Valencia, CA), digested with BamHI, and ligated into BamHI-digested and alkaline phosphatase-treated pGEX-6P-1 (Amersham Biosciences). This construct was designed to express DC3 fused to the C terminus of glutathione S-transferase (GST). The wild-type fusion protein is termed “GST-DC3” and the DC3 end mutant fusion protein is termed “GST-DC3 (E74Q, E152Q).” The constructs were transformed into Escherichia coli strain BL21 (Amersham Bioscience), and protein expression was induced with either 0.1 mM (GST-DC3 (E74Q, E152Q)) or 0.3 mM (GST-DC3 (isopropyl-β-D-thiogalactopyranoside for 2 h at room temperature. A lower concentration of isopropyl-β-D-thiogalactopyranoside was used to induce the mutant protein to aid in its solubility (according to The Recombinant Protein Handbook from Amersham Biosciences). After induction, the recombinant protein was released from the bacterial cells using a French® press (Thermo Electron Corporation, Rochester, NY). The bacterial lysate was centrifuged, and the soluble fraction was applied to a gluthathione-Sepharose 4B column (Amersham Biosciences) at 4 °C; the affinity-purified fusion protein was eluted with 50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione, 1 mM diithothreitol (DTT).

The size and purity of the recombinant protein was determined by SDS-PAGE. Protein concentration was determined with the bicinchoninic acid assay (Pierce) using bovine serum albumin as standard. Prior to determination of protein concentration, the fusion protein was dia-
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DC3 was expressed in E. coli as a C-terminal fusion with GST. A protein of the appropriate mass (~47 kDa) was produced and purified; SDS-PAGE analysis of the purified protein is shown in Fig. 3A. To determine whether bacterially expressed DC3 was biologically functional, the GST tag was removed by digesting the fusion protein with PreScission™ protease (Amersham Biosciences) followed by glutathione-Sepharose® 4B column chromatography. The probe 45Ca2⁺ GST-tagged and was therefore retained by the column; DC3 was collected in the flow through. After determining the protein concentration of DC3 alone, it was dialyzed extensively against 20 mM Tris-HCl, pH 7.5, 150 mM NaCl using a Slide-A-Lyzer® Dialysis Cassette (Pierce) with a molecular mass cut-off of 10,000 Da to remove reduced glutathione and DTT present in the column elution buffer (DTT interferes with the bicinchoninic acid assay). In some instances, the GST moiety was removed from the fusion protein by adding DTT to 1 mM and cleaving with PreScission™ Protease (Amersham Biosciences) using a Slide-A-Lyzer® Dialysis Cassette with a molecular mass cut-off of 3,500 Da to remove DTT present in the cleavage buffer. Unless indicated otherwise, DTT was added to 1 mM before using the protein in Ca2⁺ binding assays.

Electrophoretic Properties of DC3—Introduction of recombinant DC3 into Chlamydomonas cells—Samples were always collected from the second filtrate to ensure an accurate measure of the unbound Ca2⁺.

Dialysis Cassette (Pierce) with a molecular mass cut-off of 10,000 Da was used extensively at 4°C to remove CaCl2 solution containing 2 mM EDTA and 1 mM EGTA (as opposed to a measure of the unbound Ca2⁺). A 25-μl aliquot of the mixture was transferred to an electroporation cuvette and incubated at 15°C for a total of 15 min after cell washing. An electric pulse of 350 V was then applied to the mixture using an ECM600 electroporation apparatus (BTX) and a resistance of 24 ohms and a conductance of 600 microfarads. The overall time constant used for each sample was around 12 ms. After electroporation, the cuvette was incubated at room temperature for 30 min with shaking every 5 min. The cells were then washed several times in HMDKCaS (30 mM HEPES, pH 7.4, 5 mM MgSO4, 1 mM DTT, 50 mM CH3COOK, 1 mM (CH3COO)2Ca, 60 mM sucrose) and resuspended to a concentration (as opposed to a measure of the unbound Ca2⁺) and calcium (as opposed to a measure of the unbound Ca2⁺) was used to deduce the amount of calcium bound per molecule of GST-DC3.

The tryptophan fluorescence of GST-DC3 was analyzed using a SPEX fiber method of Dunahay (22), we co-transformed strain 026.2, an arg7 derivative of Chlamydomonas—By using the silicon carbide fiber method of Dunahay (22), we co-transformed strain 026.2, an arg7 derivative of Chlamydomonas—By using the silicon carbide fiber method of Dunahay (22), we co-transformed strain 026.2, an arg7 derivative of Chlamydomonas. To reduce nonspecific protein binding, the filter unit was pre-washed several times in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, with or without 1 mM DTT was placed in the upper chamber of a Centricrifugal Filter Device with a nominal molecular mass cut-off of 10,000 Da (YM-10; Millipore Corp., Bedford, MA). To reduce nonspecific protein binding, the filter unit was pre-washed several times in TAP medium supplemented with 60 mM sucrose (TAP + S), resuspended in 125 μl TAP + S, and left at room temperature for 2–3 h to allow the cells to recover.

Swimming velocity and flagellar beat frequency measurements—Swimming velocity was measured as described by Kamiya (24). Brieﬂy, motile cells were imaged using dark-field microscopy, and their swimming behavior was recorded with a CCD camera and VCR. Images from the videotape were “captured” using motion-capture software (Motion Capture, InterQuest, Osaka, Japan) at a rate of 10 frames/s. The video clips were then played on a computer monitor, and representative cells to be tracked were chosen by the operator, and their positions as a function of time were determined by cell-tracking software (Image Tracker PTV, InterQuest). The swimming velocity of ~30 cells was used to calculate the average swimming velocity for each strain. For photo-kinetics experiments, cells were dark-adapted for 2 h and then observed with or without 5 min of pre-illumination with white ﬂuorescent light. Cells without pre-illumination were imaged using red light (630-nm cut-off filter), and those with pre-illumination were imaged using white light. Again, the average swimming velocity for each strain was determined from measurements of ~30 cells.

The average flagellar beat frequency of a population of forward swimming cells was determined by fast Fourier transform analysis, exactly as described by Kamiya (25). To determine the average flagellar beat frequency change induced by photoshock, cells were imaged using a ×40 objective (instead of the usual ×10 objective) and at low cell density to permit fast Fourier transform analysis of individual cells. Forward swimming cells were initially viewed and analyzed using red light; photoshock was then induced by removal of the red filter from the observation beam, and the beat frequency during the resulting transient backward swimming was determined. About 20 cells were used to calculate the average flagellar beat frequency before and during photoshock for each strain.

Electron Microscopy—Flagellar axonesomes were isolated from cells grown in TAP medium (18) by using the method of Witman (23) and prepared for electron microscopy as described by Kamiya (24).

Electrophoretic properties of DC3—Introduction of recombinant DC3 into Chlamydomonas cells—Samples were always collected from the second filtrate to ensure an accurate measure of the unbound Ca2⁺.

Electrophoresis of recombinant DC3 into Chlamydomonas cells—Samples were always collected from the second filtrate to ensure an accurate measure of the unbound Ca2⁺.

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in close proximity (14), these results strongly suggest that they
include that Cys-66 and Cys-69 are the only two cysteine residues
involved in calcium-binding activity to the protein. Inasmuch as molecular modeling pre-
vents understanding of the Ca2+-binding loop (Fig. 2), two of
which (Cys-66 and Cys-69) are in sufficiently close proximity, they
form a disulfide bond that could block Ca2+-binding. To test this, we examined the Ca2+-binding activity of recombinant
GST-DC3 in dialysis buffer (20 mM Tris-HCl, pH 7.5, 150
mM NaCl) without the addition of DTT. Under these conditions, the
Ca2+-binding activity of the protein was completely elimi-
nated (Fig. 4A). Addition of 1 mM DTT restored Cys-69 binding
activity to the protein. Inasmuch as molecular modeling predicts
that Cys-66 and Cys-69 are the only two cysteine residues in
close proximity (14), these results strongly suggest that they
can form a redox-sensitive disulfide bridge that prevents Ca2+-
binding. These data also suggest that the first consensus Ca2+-
binding loop is responsible for the in vitro Ca2+-binding activity of DC3.

DC3 Binds Mg2+—In 1980, Reid and Hodges (27) proposed
the acid pair hypothesis that correlated Ca2+-affinity with the
location and number of paired, negatively charged chelating
residues. They predicted the highest Ca2+-affinity will occur
when an EF-hand has acidic residues in loop positions X, –X,
Z, and –Z. Studies using synthetic EF-hand peptides have
indicated that Mg2+ will bind to those motifs containing 3 or 4
acidic residues in chelating positions, as long as two of the
acidic residues are in Z and –Z positions (a “Z-acid pair”) (28,
29). Subsequently, Tikunova and colleagues (30) demonstrated
that the presence of a Z-acid pair in the first EF-hand of CaM
was essential for Mg2+-binding with a physiologically relevant
affinity.

Because EF2 has 4 acidic residues in chelating positions, two
of which form a Z-acid pair (Fig. 2), DC3 could potentially be a
Ca2+- and Mg2+-binding protein. To investigate this possibility,
we performed the calcium binding assay in the presence of
physiological (1 mM) concentrations of Mg2+ (Fig. 4B). Similar
results were obtained with DC3 after removal of the GST tag, demonstrating that calcium
binding is an intrinsic property of DC3 rather than a nonspe-
cific effect due to the GST moiety (data not shown). Scatchard
analysis of the GST-DC3 Ca2+-binding data indicated that the
calcium affinity of DC3 (expressed as a dissociation constant,
Kd) is 1.2 x 10^-5 M (Fig. 4B); similar results were obtained with
DC3 alone (Kd = 1.3 x 10^-5 M). This is in the range expected to
be physiologically relevant for photoshock (see Introduction).

The Ca2+-Binding Activity of DC3 Is Redox-sensitive—DC3
contains four cysteine residues (underlined in Fig. 2), two of
which (Cys-66 and Cys-69) are within the first consensus
calcium-binding loop. Cys-69 is one of the six loop residues (position
–Y) that coordinates the Ca2+-ion (see Introduction). If
Cys-66 and Cys-69 are in sufficiently close proximity, they
might form a disulfide bond that could block Ca2+-binding. To test this, we examined the Ca2+-binding activity of recombinant
GST-DC3 in dialysis buffer (20 mM Tris-HCl, pH 7.5, 150
mM NaCl) with the addition of DTT. Under these conditions, the
Ca2+-binding activity of the protein was completely elimi-
nated (Fig. 4A). Addition of 1 mM DTT restored Cys-69 binding
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can form a redox-sensitive disulfide bridge that prevents Ca2+-
binding. These data also suggest that the first consensus Ca2+-
binding loop is responsible for the in vitro Ca2+-binding activity of DC3.
still did not eliminate binding. These data indicate that the metal ion-binding site can bind either Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. However, because Ca\textsuperscript{2+} binding is not completely abolished even in the presence of ~200-fold excess Mg\textsuperscript{2+}, the affinity of DC3 for Ca\textsuperscript{2+} is much higher than its affinity for Mg\textsuperscript{2+}.

Effect of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} on the Fluorescence Spectra of DC3—Some EF-hand proteins that bind calcium, such as CaM, change their shape and thereby regulate cellular processes. Others bind calcium but do not change shape; these proteins may be involved in Ca\textsuperscript{2+} buffering or anchoring protein complexes (31). Single tryptophan residues in Ca\textsuperscript{2+}-binding proteins are often used as intrinsic fluorescent probes to determine protein structural changes and substrate binding. Among the properties used are changes in the fluorescence intensity and wavelength maximum (\(\lambda_{\text{max}}\)). In order for the information they provide to be meaningful, Trp residues must be located close to the metal ion-binding site. Because DC3 contains only one Trp residue (double underlined in Fig. 2), which is located in the C-terminal part of EF2 helix E, we measured the Trp fluorescence of GST-DC3 in the absence and in the presence of 1 mM CaCl\textsubscript{2} or 20 mM MgCl\textsubscript{2}. The maximal Trp fluorescence intensity decreased in the presence of either metal (Fig. 6), supporting the above finding that DC3 binds Ca\textsuperscript{2+} and providing further evidence that it also binds Mg\textsuperscript{2+}. DC3 underwent an ~1.1-fold decrease in its maximal Trp fluorescence intensity upon Mg\textsuperscript{2+} binding; binding of Ca\textsuperscript{2+} caused an ~1.2-fold decrease. The magnitude of the intensity change upon binding of either metal suggests local conformational changes within the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-binding loop (32). Binding of either metal caused a small 1-nm red shift in the \(\lambda_{\text{max}}\) of fluorescence emission from 345 to 346 nm (Fig. 6). The shift to a slightly longer wavelength suggests that Trp-60 is in a more polar environment when DC3 is in its metal-bound state.

Site-directed Mutagenesis Abolishes DC3 Ca\textsuperscript{2+} Binding—The results of our calcium binding assays indicated that DC3 contains at least one functional Ca\textsuperscript{2+}-binding site. To determine whether DC3 was binding calcium through either of its consensus sites, we introduced a point mutation into both EF2 and EF4 to change the highly conserved glutamate residue at position –Z (Fig. 2, dark and underlined) to a glutamine. This Glu to Gln mutation has been shown to abolish or greatly reduce the calcium binding of other EF-hand proteins (for review see Ref. 33). We expressed the mutant protein as a C-terminal fusion with GST; SDS-PAGE analysis of the bacterially expressed, affinity-purified mutant protein, which we named GST-DC3+E74Q,E152Q, is shown in Fig. 7. The purified protein was of the expected mass (~47 kDa). When the calcium binding potential of the modified protein was tested using the same ultrafiltration method as was used for the wild-type protein, we found that the Glu to Gln mutations completely abolished DC3 calcium binding (Fig. 8). Therefore, either EF2 or EF4 must mediate the in vitro calcium binding ability of wild-type DC3.

The EF-hand Mutant Gene Restores Outer Arms to a DC3-Null Strain—Because they are missing ~50% of their outer dynein arms, Chlamydomonas cells lacking DC3 have a reduced beat frequency and swim in a jerky manner at about one-half the speed of wild-type cells (14). We showed previously that nuclear transformation with the wild-type DC3 gene restores outer dynein arms, wild-type motility, and a wild-type photoshock response (14). To determine the role of the calcium binding activity of DC3 in vivo, we transformed a DC3-null strain with a DC3 gene containing the same Glu to Gln mutations that abolished the calcium binding activity of DC3 in vitro. If the calcium affinity in vivo of DC3 is similar to its calcium affinity in vitro (~10\textsuperscript{-5} M), the EF-hand mutant protein should not affect either ODA-DC or outer arm assembly, because flagellar assembly in Chlamydomonas presumably occurs at a steady-state calcium concentration of ~10\textsuperscript{-8} M (4).

Indeed, transformation of the DC3-null strain with the modified gene resulted in the complete restoration of outer dynein arms (Fig. 9). Moreover, the EF-hand mutant gene restored wild-type swimming in 29% of the transformants screened (45/154), a percentage comparable to that obtained by transformation with the wild-type gene (24/96 or ~25%). (Because transformed cells were selected on the basis of co-transformation with a selectable marker (ARG7; see “Experimental Procedures”), it is expected that only some of the selected strains would express DC3.)

To verify our qualitative conclusion that the mutant gene had restored wild-type motility to the DC3-null strain, we quantitatively measured its swimming speed and beat frequency and compared them to those of the original DC3-null strain and a DC3-null strain transformed with the wild-type gene. As reported above, DC3-null cells swim with an average velocity of ~130 \(\mu\text{m/s}\) and have an average beat frequency of...
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Mutant Cells—The switch from forward swimming to backward swimming is not normal, indicating that DC3 is unlikely to be the sole flagellar calcium sensor for photoshock and furthermore that the inability of the DC3-null strain to generate backward swimming is probably due to the incomplete number of outer dynein arms.

Beat Frequency Is Normal in Backward Swimming EF-hand Mutant Cells—During the photoshock response, the switch from forward to backward swimming is accompanied by an increase in flagellar beat frequency (34, 35). Because this subtle change might have been missed in our qualitative analysis of photoshock (see above), we measured the flagellar beat frequency in the wild-type and mutant strains before and during the photoshock response. The beat frequency of wild-type cells was 62 Hz during forward swimming and increased to 84 Hz when the cells were induced to swim backward during photoshock. The beat frequency of DC3-null cells rescued with the wild-type DC3 gene was 59 Hz during forward swimming and increased to 83 Hz during backward swimming. The beat frequency of EF-hand mutant cells was 58 Hz during forward swimming and increased to 80 Hz during backward swimming. Therefore, the EF-hand mutations in DC3 do not prevent the increase in flagellar beat frequency that occurs as calcium rises into the 10^{-5} M range during photoshock.

Photokinesis Is Normal in the EF-hand Mutant Strain—Photokinesis is a phenomenon in which the swimming speed of a microorganism varies as a consequence of the light stimulus intensity. Although this type of photosresponse has not been studied in detail in Chlamydomonas, Moss and Morgan (36) report that light-adapted wild-type cells swim faster than dark-adapted cells, and Pazour et al. (15) have shown that the speed of wild-type cells increases as the intensity of the light stimulus increases. Interestingly, Moss and Morgan (36) report that photokinesis does not occur in strains that lack outer dynein arms. These observations suggest that a light-activated signal transduction cascade is acting at the level of the outer dynein arms to increase cell motility. A light-activated signal transduction cascade might use cytoplasmic reducing equivalents (i.e., electrons) to transmit signals because their levels change when Chlamydomonas is in the light (37).

Because the ODA-DC is in direct contact with dynein (11), and DC3 calcium binding is redox-sensitive, the Ca^{2+} binding activity of DC3 may play a role in photokinesis. To investigate this possibility, we measured the average swimming velocity of dark-adapted versus light-adapted wild-type cells, DC3-null cells, DC3-null cells rescued with the wild-type DC3 gene, and EF-hand mutant cells. As DC3 calcium binding in vitro is abolished by mutation of the consensus EF-hands, DC3 in the EF-hand mutant strain presumably is constitutively "off," i.e. cannot bind Ca^{2+} regardless of the redox state of the cell, and will not be able to mediate photokinesis if the latter depends on...
the oxidation state of DC3. Dark-adapted wild-type cells swim with an average velocity of 197 μm/s; in the light, their average swimming velocity increased to 224 μm/s. Dark-adapted DC3-null cells had an average swimming velocity of 118 μm/s; in the light, their average swimming velocity increased to 144 μm/s. Dark-adapted DC3-null cells rescued with the wild-type DC3 and EF4 completely abolished DC3 calcium binding. These are the first quantitative data indicating that Chlamydomonas undergoes a photokinetic response; however, the redox sensitivity of DC3 calcium binding does not appear to play a role in this response.

**DISCUSSION**

In the present study, we have demonstrated that DC3, the 21-kDa subunit of the ODA-DC, has calcium binding activity. DC3 is the fourth CTER calcium-binding protein to be identified in the flagellum of Chlamydomonas. The other three CTER proteins are CaM, centrin, and LC4. CaM is an integral axonemal component (5, 6) and is associated with the radial spokes (38). Centrin is a subunit of some inner dynein arms (7). LC4 is a subunit of outer dynein arms (8). The presence of multiple calcium-binding proteins each associated with a discrete axonemal structure suggests that there are multiple sites of calcium control in the flagellum. This is consistent with the complex architecture of the axoneme, which contains multiple structures that undoubtedly must respond in a coordinated manner to changes in intraflagellar Ca2+.

Although DC3 has four EF-hand motifs, only EF2 and EF4 fit the consensus pattern for calcium binding. EF1 has a lysine residue in position Y of the calcium-binding loop. According to the PROSITE consensus pattern for calcium binding (see Introduction), only aspartate, asparagine, and serine are tolerated at this position, so EF1 is not expected to bind calcium. EF3 has two residues that deviate from the consensus, one of which is in a calcium-chelating position (position Z). Furthermore, molecular modeling of DC3 (14) predicts that EF3 forms a β-hairpin that would most likely disrupt the coordination geometry of the loop and prevent calcium binding. Our *in vitro* calcium binding assays indicate DC3 has at least one functional calcium-binding site. Site-directed mutagenesis of EF2 and EF4 completely abolished DC3 calcium binding. These data indicate that either EF2 or EF4 is responsible for the *in vitro* binding activity of DC3. Based on the redox sensitivity of DC3 calcium binding, EF2 is the more likely candidate because it contains three cysteine residues, two of which are located within the calcium-binding loop.

Many EF-hand proteins such as CaM undergo a global conformational change when they bind Ca2+ but only a local conformational change when they bind Mg2+ (32, 39). The difference between the Ca2+- and Mg2+-bound conformations can be determined by comparing the Trp emission spectra of the protein. For instance, the Trp fluorescence of CaM increases ~2.8-fold in the presence of Ca2+ yet only ~1.4-fold in the presence of Mg2+ (30). Although the maximal Trp fluorescence intensity of Ca2+-bound DC3 differed from that of Mg2+-bound DC3, the intensity change was small (~1.2-fold versus ~1.1-fold, see Fig. 6). Furthermore, Ca2+- binding to CaM induces a small blue shift in λmax whereas Mg2+- binding induces a small red shift (30). With DC3, the position of λmax was the same in the presence of either metal. Taken together, these data indicate that although there are clearly two conformational states of DC3 depending on which metal is bound, the structural difference is minor when compared with that of CaM (30).

As mentioned above, a large shift in the intensity of Trp fluorescence occurs when some EF-hand proteins bind calcium and is indicative of extensive conformational changes (32). The slight fluorescence intensity shift of Ca2+-bound DC3 versus Ca2+-free DC3 (Fig. 6) suggests that calcium binding to DC3 does not cause major structural effects (32). Other examples of EF-hand proteins that bind calcium yet change shape only slightly include the regulatory domain of cardiac TnC (40, 41) and the calcium signal modulator, calbindin D9k (for review see Ref. 42). These proteins have non-polar amino acids in key regulatory positions, specifically a cysteine (Cys-84) in cardiac TnC and an isoleucine (Ile-75) in calbindin D9k (43). Proteins that are CaM-like have buried polar residues in the structurally analogous positions. It is believed that these buried polar residues are critical for generating a large conformational change (reviewed in Ref. 42 and see Ref. 43). In fact, replacing the polar lysine residue with a non-polar isoleucine residue (K75I) in the N terminus of CaM prevents the protein from undergoing a large conformational change (43). DC3 has a non-polar cysteine residue (Cys-82) in the structurally analogous position, lending support to our interpretation that metal-ion binding to DC3 induces only a small conformational change.

*In vivo*, DC3 is in a complex with DC1 and DC2 (11); therefore, it is conceivable that DC1 and/or DC2 exert a substantial effect on the structure of DC3. For instance, DC3 may significantly change conformation upon DC1 and/or DC2 binding. This mode of structural change would be analogous to that of cardiac TnC, which does not significantly change conformation upon Ca2+ binding yet does significantly change conformation upon TnI binding (44). Binding of TnI to TnC also increases the Ca2+ sensitivity of TnC ~10-fold (31). Likewise, the presence of CaM-dependent enzymes or short peptides corresponding to CaM-dependent enzyme-binding sites increases the Ca2+ affinity of CaM (45, 46). These data indicate the calcium binding activity of DC3 in complex with DC1 and DC2 may be higher than the calcium binding activity of the purified protein.

The above studies highlight the important role that protein interactions have in determining the conformation and Ca2+-binding properties of EF-hand proteins. This was clearly demonstrated by experiments in which target binding restored Ca2+-binding site mutants of CaM (47, 48). In these studies, the binding of mutant CaMs to peptides representing CaM-binding sites changed the conformation of the mutant CaMs back to that of the wild-type protein so that they could still bind calcium and activate target enzymes. Therefore, although the site-directed mutagenesis of DC3 effectively eliminated calcium binding *in vitro* (Fig. 8), it is possible that the mutations are suppressed *in vivo* when DC3 binds to either DC1, DC2, or some other flagellar protein. Moreover, there are several examples of non-canonical EF-hands binding calcium when their structures are stabilized by interacting proteins (reviewed in Ref. 49); this may also be the case with DC3. Regardless of the mechanism, if mutant DC3 binds calcium *in vivo*, this may explain why no deleterious effect on photoshock was observed in the DC3-null strain that was rescued with the EF-hand mutant gene.

Alternatively, another calcium-binding protein may be the flagellar calcium sensor for photoshock. As mentioned above, CaM, centrin, and LC4 have all been localized to the axoneme. One or more of these proteins (or a yet unidentified calcium-binding protein) acting alone or in concert may initiate photoshock by relaying a message to the outer dynein arms when the intraflagellar calcium concentration rises above pCa 6. The presence of proteins with redundant functions would ensure that the cell could respond to bright light even if one of the other sensor proteins were unable to function.

Our finding that the *in vitro* calcium binding activity of DC3...
DC3 Is a Redox-sensitive Calcium-binding Protein

Kamiya, R., and Okamoto, M. (1985) demonstrated that the ATPase activity of the dox state of DC3. Intriguingly, Harrison appropriately positioned to interact with and regulate the re-association with the outer arm (11), these light chains may be chains, respectively (37, 50). Because the ODA-DC directly disulfides would have to be reduced. A class of proteins known to comprise the calcium-binding loop in a conformation that prohibits ac-

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DC3, the Smallest Subunit of the *Chlamydomonas* Flagellar Outer Dynein Arm-docking Complex, Is a Redox-sensitive Calcium-binding Protein

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