The Ca$^{2+}$/Calcineurin-Regulated cup Gene Family in Dictyostelium discoideum and Its Possible Involvement in Development

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Received 26 September 2003/Accepted 13 November 2003

Changes in free intracellular Ca$^{2+}$ are thought to regulate several major processes during Dictyostelium development, including cell aggregation and cell type-specific gene expression, but the mechanisms involved are unclear. To learn more about Ca$^{2+}$ signaling and Ca$^{2+}$ homeostasis in this organism, we used suppression subtractive hybridization to identify genes up-regulated by high extracellular Ca$^{2+}$. Unexpectedly, many of the genes identified belong to a novel gene family (termed cup) with seven members. In vegetative cells, the cup genes were up-regulated by high Ca$^{2+}$ but not by other ions or by heat, oxidative, or osmotic stress. cup induction by Ca$^{2+}$ was blocked completely by inhibitors of calcineurin and protein synthesis. In developing cells, cup expression was high during aggregation and late development but low during the slug stage. This pattern correlates closely with reported levels of free intracellular Ca$^{2+}$ during development. The cup gene products are highly homologous, acidic proteins possessing putative ricin domains. BLAST searches failed to reveal homologs in other organisms, but Western analyses suggested that Cup-like proteins might exist in certain other cellular slime mold species. Localization experiments indicated that Cup proteins are primarily cytoplasmic but become cell membrane-associated during Ca$^{2+}$ stress and cell aggregation. When cup expression was down-regulated by antisense RNA, the cells failed to aggregate. However, this developmental block was overcome by partially up-regulating cup expression. Together, these results suggest that the Cup proteins in Dictyostelium might play an important role in stabilizing and/or regulating the cell membrane during Ca$^{2+}$ stress and/or certain stages of development.

In the presence of a food source (usually bacteria), amoebae of the eukaryotic microorganism Dictyostelium discoideum grow and divide as single cells. Upon starvation, however, cell division ceases and the amoebae aggregate in response to endogenously generated waves of extracellular cyclic AMP (cAMP) into a multicellular mound. During aggregation and in the mound, the cells differentiate into two major types, prestalk and prespore cells, which sort to the anterior (−20%) and posterior (+80%) regions of the motile slug, respectively. The cells then continue to differentiate and to proceed through a variety of morphogenetic stages (termed culmination) to form (after ~24 h) a fruiting body consisting of a cluster of spores atop a long slender stalk (28).

Several direct and indirect observations suggest that changes in the concentration of free intracellular Ca$^{2+}$ ([Ca$^{2+}$]) might participate in the regulation of differentiation and/or morphogenesis during Dictyostelium development. First, direct [Ca$^{2+}$]$_i$ measurements using aequorin, Ca$^{2+}$-sensitive fluorescent dyes, or $^{45}$Ca$^{2+}$ have revealed changes during specific stages of development. For example, [Ca$^{2+}$]$_i$ has been reported to increase during the aggregation and culmination stages and to decrease during the slug stage (44). Moreover, in aggregating cells and in slugs, [Ca$^{2+}$]$_i$ is elevated by pulses of cyclic AMP which mimic the natural signals (33, 36, 44, 54). In slugs, [Ca$^{2+}$]$_i$ is substantially higher in prestalk cells than in prespore cells (1, 10, 44). Second, several dozen Ca$^{2+}$-binding proteins possessing one to five EF hand domains have been identified in Dictyostelium (e.g., 15, 37, 43; B. Coukell, unpublished data) including two calmodulins (21, 41), calcineurin B (2), and several cytoskeleton-binding proteins (e.g., see references 13 and 52). Most of the genes encoding these proteins are developmentally regulated, and some are expressed specifically in prestalk or prespore cells (43). Finally, by using drugs which are thought to alter [Ca$^{2+}$]$_i$, intracellular Ca$^{2+}$ has been implicated in cell aggregation (chemotaxis) (16, 40, 49; but see reference 48 for a contrary view), in the expression of certain prestalk and/or prespore genes (4, 40, 45, 50), and in the induction of prestalk cells (3, 27). Despite these observations strongly suggesting a role for [Ca$^{2+}$]$_i$ in Dictyostelium development, the precise Ca$^{2+}$ signaling pathways involved remain obscure.

Although [Ca$^{2+}$]$_i$ is a major regulator in eukaryotic cells, high sustained levels of intracellular Ca$^{2+}$ can be toxic. As a result, organisms have evolved a variety of mechanisms to regulate Ca$^{2+}$ homeostasis. For example, in Saccharomyces cerevisiae, cytosolic Ca$^{2+}$ levels are kept low by the concerted action of the vacuolar H$^+$/Ca$^{2+}$ exchanger, Vcx1p, and the Ca$^{2+}$-ATPases, Pmc1p and Pmr1p, associated with the vacuole and Gogli complex, respectively (11). Under conditions of Ca$^{2+}$ stress, genes encoding the two Ca$^{2+}$ pumps, as well as many other proteins, including some not directly involved in ion regulation, are up-regulated by the elevated [Ca$^{2+}$]$_i$, via the calcineurin-Crz1p/Tcn1p signaling pathway (32, 53). Stressing yeast cells with high extracellular Ca$^{2+}$ is a very effective means of identifying genes up-regulated by changes in [Ca$^{2+}$]$_i$ (53). Previously, we identified, cloned, and characterized the gene...
\( \textit{patA} \) in \textit{Dictyostelium}, which encodes a homolog of PMC1p, PatA (previously termedPAT1), a component of the contractile vacuole (34). Like PMC1p in yeast, PatA expression in \textit{Dictyostelium} is up-regulated by Ca\(^{2+}\)/calciuretin, and it is thought to function in Ca\(^{2+}\) homeostasis (35). To identify additional proteins involved in Ca\(^{2+}\) signaling and/or Ca\(^{2+}\) homeostasis in \textit{Dictyostelium}, we have used the relatively new technique suppression subtractive hybridization (SSH) (14) to select cDNAs up-regulated in cells exposed to high exogenous Ca\(^{2+}\). As a result, we have identified and characterized a novel family of Ca\(^{2+}\)-up-regulated genes (\textit{cup}) which might be involved in \textit{Dictyostelium} development.

**MATERIALS AND METHODS**

**Chemicals.** Cyclosporine A (CsA) was obtained from Calbiochem, while cyclosporine H (CsH) and FK506 were gifts of Novartis Pharma, Inc., and Fujisawa USA, respectively. Genetin (G418) was purchased from ICN Biochemicals, and cycloheximide was from Sigma.

Strains: E. coli and \textit{Ca.\,alvei} (100 mg/ml) were prepared in dimethyl sulfoxide, and cycloheximide (25 mg/ml) was prepared in water.

**Organisms, growth and development.** Most experiments were performed with \textit{D. discoideum} strain AX2 grown axenically to \(5 \times 10^{10} \) to \(10 \times 10^{10} \) cells/ml in H1.5 medium (39) or in 2-(\(N\)-morpholino)ethanesulfonic acid (MES)-H1.5 medium (35) supplemented with dihydrostreptomycin (200 \(\mu \)g/ml) and, where indicated, G418 (5 to 60 \(\mu \)g/ml). In one set of experiments, the following cellular slime mold species were also used: \textit{Dictyostelium mucoroides} strain 11; \textit{Polypodiumium violaceum} strain 1; \textit{Polypodiumium pallidum} strain 2 (5); and \textit{Dictyostelium lacteum}, \textit{Dictyostelium caveatum}, and \textit{Acetomyctostelium leptosomum} (a kind gift of R. Mutzel, Freie Universität, Berlin, Germany). The first three organisms were grown on SM agar plates with \textit{Klebsiella pneumoniae} grown on SM agar plates with \textit{fl} Mutzel, Freie Universität, Berlin, Germany. The other two strains were cultured in water.

**Materials and methods.** The three plasmids were transformed separately into AX2 cells by electroporation (39), and transformants were selected in H5 medium containing 5 to 10 \(\mu \)g of G418/ml. The G418 concentration was then increased gradually to 20 \(\mu \)g/ml for the overexpressing strains (cupA-OE and cupB-OE) and to 60 \(\mu \)g/ml for the antisense strain (cupB-AS). Production of CupA-\( \alpha \) antibodies. To produce a protein corresponding to the \(N\)-terminal 394 amino acids of CupA, the \(cupA\) cDNA was amplified by the PCR (8) using the sense primer cupeA-AS, 5'-GCCGGCCGCAAAACCATATTAGAAGAT-3' with a SacI site, and the antisense primer cupeA-AS, 5'-GCCGCTCCGTGAAGATTAATATTGAAGAT-3' with a SacI site, and the resulting transformants were analyzed in duplicate by colony hybridization.

**Northern and Western blotting.** For Northern blot analysis, total RNA was isolated from frozen cell pellets, fractionated on 1% agarose-0.66% formaldehyde-gel (20 \(\mu \)l/g), transferred to Hybond C nylon membranes (Du Pont), hybridized, and washed at high stringency as described previously (6). In most experiments, the probe was the 
\( \text{5'--3'} \) cDNA insert of \( \text{SSH clone 1} \) labeled with \( \text{\(\beta\)}\) by random priming. This sequence corresponds to nucleotides 285 to 865 of the cupc coding region, and it is 99% identical to all of the other cup genes, except \( \text{cupA} \) (97%). In initial screening experiments, cDNA inserts of the SSH clones were used as probes. For Western blot analysis, frozen cell pellets (1 \( \times 10^{7} \) to 2 \( \times 10^{7} \) cells) were lysed by gentle vortexing in ice-cold LB (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu \)g of E-64 (Sigma) per ml [pH 8.0]). Samples of cell extracts were fractionated on SDS–7.5% or –10% polyacrylamide gels, transferred to nitrocellulose, and probed with CupA-\( \alpha \) antibodies (1:750) as described previously (34).

**Regulation of cup gene expression.** For \( \text{Ca}^{2+}\) induction, late-log-phase AX2 cells in MES–H5.5 medium were harvested by centrifugation (300 \( \times \) g for 2 min), resuspended to 0.5 \( \times 10^{7} \) to \( 2 \times 10^{7} \) cells/ml in MES–H5.5, shaken in a gyratory shaker at \(-22^\circ\)C, and treated with 40 to 80 \( \mu \)m NaCl for 2 h (Northern analysis) or 4 h (Western analysis). Cycloheximide, CsA, CsH, and FK506, when present, were added 5 min before the CaCl\(_2\) to the final concentrations used in earlier studies (6, 23, 35). Cells were collected by centrifugation, washed twice in SS (10 mM NaCl, 5 mM KCl, 2.7 mM CaCl\(_2\)), frozen in dry ice-ethanol, and stored at \(-80^\circ\)C. To examine other treatments for cup induction, cells in MES–H5.5 were treated as described above with 80 mM MgCl\(_2\), MnCl\(_2\), or CoCl\(_2\) or 100 mM NaCl (ionic stress), 400 \( \mu \)M sobitol (osmotic stress) (36), 1 mM H\(_2\)O\(_2\) (oxidative stress) (18), or 30°C (heat stress) (29). In all experiments, the cells were examined microscopically for damage at the time of sampling: no lysis or other cell damage was ever detected. For cell fractionation experiments, AX2 cells were shaken in MES–H5.5 medium with 50 \( \mu \)m CaCl\(_2\) for 4 h, harvested, washed twice in SS, and resuspended to \( 5 \times 10^{7} \) cells/ml in LB (without NP-40) or in the same buffer plus 5 \( \mu \)m CaCl\(_2\). The cells were disrupted by freeze-thaw lysis, centrifugation at 4°C for 15 min, the supernatant was removed, and the pellets were resuspended to the original volume in the same lysis buffer (ice-cold). Extracellular proteins were precipitated with either 8
in BLAST searches of DNA and protein databases. All of the inserts were sequenced, and the sequences were used to identify (9,000 g for 15 min) in the same solution, and solubilized in LB. Indirect immunofluorescence. AX2 and cupB-OE cells were grown to 2 × 10^6 to 4 × 10^6 cells/ml in MES-HL5 medium. In some experiments, the AX2 cells were treated with 50 mM CaCl₂ for 4 h. The cells were washed twice in 17 mM phosphate and resuspended in the same buffer to ~3 × 10^6 cells/ml, and the cells in 100 μl were permitted to attach to cover slips for 60 min. To examine aggregating cells, AX2 cells were permitted to stream onto cover slips under buffer (23). In most experiments, the cells were fixed in 2% paraformaldehyde, washed in PBS for 20 min before staining. Immunostained cells were examined using a Bio-Rad MRC-600 confocal microscope as described previously (34).

RESULTS

Identification of the cup gene family. To identify genes in *D. discoideum* up-regulated by Ca²⁺ stress, we used the enrichment technique SSH (see Materials and Methods). Two hundred and eight clones from the subtracted library were screened by differential colony blotting, and inserts in plasmids from 41 possible positive colonies were excised and used in Northern blot analyses to probe total RNA from Ca²⁺-treated and -untreated cells. Despite using a selective procedure, most of the probes detected mRNAs expressed at approximately the same levels in the two cell populations (e.g., Fig. 1, clone 13). However, 10 of 41 inserts detected transcripts that were up-regulated in the Ca²⁺-stressed cells (e.g., Fig. 1, clones 24 and 1). These inserts were sequenced, and the sequences were used in BLAST searches of DNA and protein databases. All of the sequences identified *Dicyostelium ORFs*, but except for one sequence corresponding to *patA*, the predicted genes/proteins were either unknown or only weakly related to known genes/proteins. Interestingly, five of these sequences (including the insert in clone 1) identified the same group of cDNAs (Japanese cDNA Project) and contigs (Genome Sequencing Projects), but with slightly different percent identities. This result suggested that we might be dealing with a new gene family. An extensive analysis of cDNA and genomic DNA databases identified seven different genes. These genes were distinguished by comparing sequences in their 5' and 3' untranslated sequences, as well as in their coding regions, and by their physical relationship to other genes in the genome. Although these genes are quite large (most ORFs are ~2.3 kb), they do not contain introns. These genes have been designated Ca²⁺ up-regulated (cup) genes *A* to *G*. Their accession numbers, representative cDNA clones, and corresponding deduced proteins (CupA to -G) are given in Table 1.

Characterization of the cup proteins. The cup proteins have predicted molecular masses of 70 to 85 kDa (Table 1), except for CupA (47 kDa, see below). The N-terminal halves of the proteins are highly homologous to one another (>80% identity) (Fig. 2B), and the C-terminal halves, while more variable in length and primary structure, still exhibit a high degree of identity (data not shown). These proteins are also quite acidic, with pIs of ~4.7. SMART version 3.1 (http://smart.embl-heidelberg.de/) analysis of the proteins predicted that all, except CupE, possess two ricin domains (ricin I and II) in their N-terminal halves; CupE is predicted to lack the ricin II domain, possibly due to the small deletion and distinct amino acid sequence in this region (Fig. 2A and B). The ricin domain is named after the carbohydrate-binding (galactose) domain in the B subunit of the poisonous lectin, ricin, from *Ricinus communis* (castor bean) (22, 42). Like most other ricin domains, ricin I and II of the Cup proteins each possess three weakly homologous subdomains (Ia to -e and IIA to -c) of about 40 amino acids each with the signature motif Q-X-W near the C terminus of the sequence. In the ricin IIC subdomain, the motif is shortened to OW (or QF in CupG) (Fig. 2B). Despite the presence of these ricin-like domains, we have been unable thus far to demonstrate the binding of pure *Dicyostelium*-expressed recombinant CupB to either Sepharose 4B (Amersham-Pharmacia) or immobilized β-galactose (Pierce). In addition to the ricin domains, CupB to -G (and CupA in ORF-2, see below) are also predicted to possess a coiled-coil structure in the C-terminal halves of the proteins in the region around amino acids 480 to 510 (Fig. 2A).
Unexpectedly, when we attempted to translate the cup cDNA in clone SLA649, a TGA stop codon (followed by many others) was encountered near the middle (base pairs 1270 to 1272) of the expected ORF (ORF-1). This nonsense codon was not the result of an error in sequencing because the cDNA was sequenced on both strands and the TGA was also present in the corresponding gene, cupA. As a result of this TGA, the predicted CupA protein should contain only 394 amino acids (47 kDa; Table 1). However, when the cupA cDNA was translated from just before the TGA in a 1 reading frame, a second ORF (ORF-2) was identified extending to near the end of the cDNA insert. ORF-2 encodes a protein similar in se-

FIG. 2. (A) Domain structure of the Cup proteins. Locations of the two ricin domains (I and II) and the coiled-coil region (cross-hatched) in most of the Cup proteins are indicated. (B) Amino acid sequences of the ricin domains in the Cup proteins (CupA to -G) and the hypothetical Dictyostelium protein AAO51776 (51776) (see Discussion). Ricin I and II domains are boxed, and the approximate boundaries of the subdomains (a to c) are indicated. The signature Q-X-W motifs are indicated by asterisks. Amino acids in each protein are shown on the left. For AAO51776, only amino acids in the putative ricin domain are presented. Identical amino acids at each site are shown with white backgrounds, while similar and different amino acids have gray and black backgrounds, respectively.
quence to the C-terminal halves of CupB to -G (Fig. 3A). This finding suggested that both full-length and truncated versions of CupA might be produced in vivo. To determine the size of CupA, Dictyostelium strains were constructed that overexpressed either CupA or CupB (see Materials and Methods). As shown in Fig. 3B, the level of cup mRNA produced by the two strains was approximately the same. On the other hand, the abundance of CupA produced was much lower than that of CupB, and the mass of CupA was only about one-half that of CupB, i.e., the size of the predicted truncated CupA protein (Fig. 3C; Table 1). Under these experimental conditions, no full-length CupA protein was detected (Fig. 3C, cupA-OE) and no truncated immunoreactive peptide with a mass of 47 kDa was observed in extracts of Ca2+-treated AX2 cells (Fig. 3C, AX2+).

**Regulation of cup expression.** Since preliminary work indicated that cup expression is induced by high concentrations of extracellular Ca2+, further experiments were performed to examine the kinetics, specificity, and mechanism of cup mRNA and protein up-regulation. As shown in Fig. 4A, when vegetative AX2 cells were incubated with 50 mM CaCl2, cup mRNA up-regulation was detectable after 1 h and maximal by 2 h. This is somewhat faster than the up-regulation of patA (35), but both processes exhibited a similar Ca2+ dose response (Fig. 4B; 35). In different experiments, the magnitude of cup mRNA up-regulation by 40 mM (or higher) Ca2+ was usually 30- to 40-fold. Like patA regulation (35), cup mRNA induction and protein induction were dependent on both protein synthesis and a functional calcineurin. Addition of the protein synthesis inhibitor cycloheximide (500 μg/ml) or the calcineurin inhibitor CsA (33 μg/ml) or FK506 (30 μg/ml) 5 min before the addition of 50 mM CaCl2 completely inhibited the synthesis of cup message and protein. In contrast, addition of CSH (33...
FIG. 5. Regulation of cup gene expression during development. (A) Northern blot analysis of cup expression in cells developing on PBS agar. Nonaxenically grown AX2 cells were washed free of bacteria and plated on PBS agar at a density of 3.2 × 10^6 cells/cm^2. The plates were incubated in a humid environment at 22°C, and total RNA was isolated from cells at 3-h intervals. Developmental timing: 0 h, vegetative cells; 6 to 9 h, aggregation; 12 h, tipped aggregates; 15 h, slugs; 18 to 21 h, culmination; 24 h, fruiting bodies. (B) Effect of Ca^2+ on cup expression during development. Nonaxenically grown AX2 cells were washed free of bacteria in 20 mM MES–NaOH (pH 6.6) and plated at the same density as in panel A on agar prepared in the same MES buffer with or without 20 mM CaCl_2. At the times indicated, total RNA was isolated from cells and used in Northern blot analysis. The developmental timing under these conditions was approximately the same as on PBS agar. Northern blots were probed with the cDNA insert from SSH clone 1.

μg/ml), a nonimmunosuppressant analog of CsA, had no effect (Fig. 4C and D). In addition to the effects of CsA and FK506 on cup expression, overexpression of the Dicyostelium homolog of the yeast calcineurin regulator, Rcn1p (24), also strongly suppressed the up-regulation of Cup and PatA expression by high Ca^2+ concentration (R. Jouliae and B. Coukell, unpublished data). It can be seen in Fig. 4D that on Western blots, the Cup proteins usually resolve into three (occasionally four) bands, with two heavy bands of larger mass and one weaker band of smaller mass. This pattern is consistent with the predicted sizes of the Cup proteins (Table 1). To determine if cup up-regulation responds only to Ca^2+ stress, the amoebae were exposed for 2 h to the following conditions: 100 mM NaCl (data not shown) or 80 mM MnCl_2, MgCl_2 (data not shown) or CoCl_2 (ionic stress), 400 mM sorbitol (osmotic stress), 1 mM H_2O_2 (oxidative stress), or 30°C (heat stress). Of these treatments, only MnCl_2 up-regulated the Cup proteins (Fig. 4E). Mn^2+ ions had a similar stimulatory effect on patA expression (35). Like Ca^2+, Mn^2+ probably acts through calcineurin because this ion has been reported previously to partially activate this protein phosphatase (25). Similar results were obtained when the stressed cells were examined by Northern blot analysis (data not shown). Together, these results suggest that like patA up-regulation, induction of the cup genes is Ca^2+/calcineurin and protein synthesis dependent.

To determine if cup expression is regulated during development, AX2 cells were grown with K. pneumoniae, washed free of bacteria, and permitted to develop on PBS agar plates. Under these conditions, the level of cup message increased during the aggregation phase, decreased during the mound/slug phase, and increased again during culmination (Fig. 5A). Careful examination of these Northern blots revealed the existence of several very closely spaced transcripts which were not resolved on RNA blots of Ca^2+-stressed cells. A similar developmental pattern was observed for Cup proteins by Western blotting (data not shown). To examine the effects of Ca^2+ on cup expression during development, the cells were developed on MES-buffered agar plates without and with 20 mM CaCl_2 (Fig. 5B). In the absence of Ca^2+, cup mRNA expression showed the same developmental pattern as on PBS agar, i.e., high early and late and low during the slug phase. The presence of Ca^2+-up-regulated cup expression substantially during early aggregation (5 h) and at the mound stage (10 h), but it had no effect during later stages of development (15 and 20 h). This result is probably due to the fact that at later times the developing structures are raised off the agar and the cells are not exposed directly to the Ca^2+. This observation suggests further that the increased cup expression during late development in the absence of Ca^2+ is likely due to internal rather than to external factors.

**Cellular localization of the Cup proteins.** To understand the function of the Cup proteins, we first examined their cellular localization by indirect immunofluorescence and by cell fractionation. In untreated vegetative AX2 cells, CupA-N antibody staining was weak and diffuse throughout the cells (Fig. 6A, panel a). When the cells were Ca^2+ stressed with 50 mM CaCl_2 for 4 h, the intensity of staining increased dramatically. Most of this staining was also distributed throughout the cells (Fig. 6A, panel b); however, in cells less intensely stained, a broad band of staining was frequently observed associated with the cytoplasmic side of the cell membrane (Fig. 6A, panel c). A similar staining pattern was seen in vegetative cells overexpressing the CupB protein (Fig. 6A, panel d) but not in cells overexpressing the truncated CupA protein (data not shown). Interestingly, when AX2 cells aggregating under buffer were fixed and examined, the staining in a majority of the cells was confined largely to a region around the periphery of the cells. Moreover, within this band of staining, foci of more intense staining could be detected (Fig. 6A, panel e). The Cup proteins in aggregating cells are not components of cytoskeletons isolated and analyzed as described previously (13; data not shown). To determine if Cup proteins are also present on the exterior surface of CupB-OE cells, the cells were fixed without permeabilization and stained (see Materials and Methods). No fluorescence was detected on these cells; however, fluorescence was intense (especially around the inner border of the cells) if the same cells were fixed and permeabilized before staining (data not shown). The intracellular staining patterns of the paraformaldehyde- and glutaraldehyde-fixed cells were very similar. To determine if the Cup proteins are in the soluble or insoluble fractions of the cell, and if they are secreted, AX2 cells were treated with Ca^2+, disrupted by freeze-thaw lysis in the presence of EDTA or Cu^2+, and fractionated by centrifugation, and the soluble and insoluble components were analyzed by Western blotting. Proteins in the incubation medium were also analyzed after being concentrated by precipitation (see Materials and Methods). Under these conditions, the Cup proteins were found exclusively in the soluble fraction regardless of the presence or absence of Ca^2+, and there was no evidence of secretion (Fig. 6B). Together, these results suggest that the Cup proteins are primarily cytosolic or weakly bound...
to intracellular membranes, but under certain conditions, they can become associated with the inner surface of the plasma membrane.

**Antisense RNA inhibition of cup expression.** To determine if the Cup proteins are necessary for Dictyostelium growth and/or development, an attempt was made to inhibit cup expression by overexpressing antisense cupB RNA. Since the sequences of the seven cup genes are very similar (especially at the 5’ ends), it was hoped that it might be possible to down-regulate all of the genes. To do this, the complete ORF of the cupB cDNA was cloned in an antisense orientation into the Dictyostelium expression vector pDXA-HC, and it was transformed into AX2 cells (see Materials and Methods). Induction of Cup protein expression by Ca\(^{2+}\) was then examined by Western blotting extracts of the antisense transformants and cells transformed with vector alone (control). As shown in Fig. 7A, Cup proteins were up-regulated normally in the control cells but very little protein could be detected in the antisense cells. (Actually a trace of Cup protein was visible on the original blot, but it was estimated to be less than 10% of the protein in the control.) Similar results were obtained by Northern blotting using a cupB sense-specific RNA probe (data not shown). During continuous culturing over the next few weeks, it was noticed that the degree of cup repression in the antisense cells decreased; i.e., more Cup protein was up-regulated in response to Ca\(^{2+}\).

The growth rates of control and antisense cells were comparable under both axenic and nonaxenic conditions (data not shown). But, when the two strains were plated on MES agar, the control cells developed normally while the antisense cells failed to aggregate, even after 48 h (Fig. 7B, panels a and b). Interestingly, this developmental defect in the antisense cells was partially suppressed by the presence of 20 mM CaCl\(_2\) in the agar (Fig. 7B, panel d). Moreover, the degree of suppression of this defect appeared to be directly related to the magnitude of Cup protein up-regulation. For example, cells capable of synthesizing little Cup protein produced only small, misshapen developmental structures (results not shown) while cells producing more Cup protein formed more normal-looking fruiting bodies, although many cells were not incorporated and remained on the substratum (shown in panel d). These results suggest that the Cup proteins might be necessary for normal development.

**Possible Cup-like proteins in other cellular slime mold species.** BLAST searches of DNA and protein databases failed to identify sequences in other organisms related to the cup genes or proteins, except in the regions of the ricin domains. Since Cup protein sequences in the N-terminal half are very highly conserved, we decided to use the CupA-N antibodies to screen
other cellular slime mold species for Cup-like proteins. Our criteria for Cup-like proteins were (i) a family of proteins with masses similar to the Cup proteins, and (ii) they should be up-regulated by exogenous Ca\(^{2+}\). As representative species, we chose organisms corresponding to the major branches of the cellular slime mold phylogenetic tree (T. Winkler and R. Mutzel, unpublished data). When amoebae of the various species were incubated under our standard induction conditions for *Dictyostelium* (see Materials and Methods) and analyzed by Western blotting, three species showed protein patterns which satisfied our criteria (Fig. 8). *P. violaceum* cells up-regulated four proteins somewhat smaller than the Cup proteins while *D. lacteum* and *D. mucoroides* (data not shown) up-regulated at least three proteins similar in mass to the Cup proteins. The protein bands associated with *D. mucoroides* extracts were very weak and not seen in every experiment. In addition to these species, the two evolutionarily related species, *P. pallidum* (data not shown) and *A. leptosomum* (Fig. 8), produced proteins often appearing as a doublet about the same size as the Cup proteins. However, these proteins were not up-regulated by Ca\(^{2+}\) under the conditions employed. Finally, no Cup-like proteins were ever detected in *D. caveatum* (not shown). Based on this preliminary analysis, the results suggest that at least some other cellular slime mold species might produce Cup-like proteins.

**DISCUSSION**

We employed the SSH technique to identify genes in *D. discoideum* which are up-regulated by exposure to high environmental Ca\(^{2+}\) concentrations. Of the 208 clones from the subtracted library that we analyzed, only 10 contained cDNAs corresponding to genes up-regulated more than fivefold by the Ca\(^{2+}\) treatment, and only one of these genes (*patA*) had a previously known function. Although initially surprising, results of a recent genome-wide analysis of gene expression regulated by the Ca\(^{2+}\)/calcineurin/Crz1p signaling pathway in *S. cerevisiae* (53) suggest that these findings might not be unreasonable. In the yeast study, 125 genes were up-regulated by high Ca\(^{2+}\) concentration but only 14 (11.2%) were increased more than fivefold. Moreover, even in this thoroughly studied organism, ~40% of the 125 genes had no known function. Since *Dictyostelium* appears to have a calcineurin signaling system similar in many respects to *S. cerevisiae* (35; R. Joulaie and B. Coukell, unpublished data), it is possible that this organism might also possess a large number of genes involved in a variety of processes that are subject to Ca\(^{2+}\) regulation.

Interestingly, five of the clones isolated by SSH contained cDNAs corresponding to members of the novel *cup* gene family and a detailed examination of cDNA and genomic DNA databases revealed seven *cup* genes (*cupA* to -*G*; Table 1). A variety of observations suggest that most (perhaps all) of the *cup* genes are expressed in *Dictyostelium* cells and are up-regulated by Ca\(^{2+}\). First, all of the genes except *cupA* (see below) have a complete ORF. Second, cDNAs corresponding to six of the genes, including *cupA*, are present in the cDNA database (Table 1); cDNAs corresponding to the seventh gene (*cupD*) might also be present, but the sequences of the genes are so similar, it is difficult to be sure. Finally, Western blot analysis of Ca\(^{2+}\)-stressed cells revealed three or four closely spaced bands, including two heavy bands which might contain multiple proteins (e.g., see Fig. 3C and 4D). This possibility of
overlapping proteins on the gel is supported by the predicted masses of the Cup proteins (Table 1). Results of the kinetic and drug sensitivity experiments (Fig. 4A to D) suggest that Ca^{2+} up-regulation of the cup genes, like that of patA (35), is mediated by a pathway involving calcineurin and protein synthesis. The requirement for protein synthesis probably accounts for the relatively slow induction of these calcineurin-dependent genes in Dicyostelium (1 to 2 h) compared to S. cerevisiae (15 min) (53). In mammalian cells and in S. cerevisiae, Ca^{2+}/calcineurin is thought to dephosphorylate cytosolic transcription factors of the NFAT family and Crz1p/Tcn1p, respectively, leading to their nuclear localization and direct binding to specific regulatory sequences in calcineurin-dependent genes (reviewed in reference 9). The fact that cup and patA up-regulation by Ca^{2+} is also dependent on protein synthesis suggests that Ca^{2+}/calcineurin signaling in Dicyostelium might involve a somewhat different mechanism. In S. cerevisiae, certain subsets of calcineurin-dependent genes are activated by environmental conditions other than high Ca^{2+} concentration, including exposure to Na^+, H_2O_2, sorbitol, and heat (19, 53). However, the cup genes in Dicyostelium were up-regulated primarily by Ca^{2+}, and to lesser degree by Mn^{2+} (Fig. 4E). Therefore, the cup proteins would appear not to be general stress factors.

All Cup proteins are predicted to possess at least one ricin domain (I), and most have two ricin domains (I and II), in their N-terminal halves (Fig. 2A). Analysis by the SMART program indicates that domain I in all proteins exhibits higher homology to ricin domains in other proteins than domain II, probably due to the modification of the third Q-X-W motif in region IIc (Fig. 2B). Although many proteins with ricin domains have been shown to bind galactose-containing carbohydrates, our attempts to demonstrate binding of pure recombinant CupB to immobilized galactose and to Sepharose 4B have been unsuccessful. This is unlikely due to incorrect folding of the recombinant protein because it was expressed in Dicyostelium cells. It is possible that the Cup proteins bind a Dicyostelium-specific carbohydrate or glycoprotein which has not yet been identified. Alternatively, despite the Q-X-W motifs and other features of ricin domains, the ricin-like structures in the Cup proteins might bind molecules other than carbohydrate. Although this is the first description of ricin domains in Dicyostelium, another protein (GenBank accession no. AA051776) possessing a ricin domain near its N terminus was identified recently during the sequencing and analysis of chromosome 2 of this organism (20). This hypothetical 691-amino-acid protein is ~30% identical to CupB along its length. Interestingly, the ricin domain in this protein is in a region comparable to domain II in the Cup proteins and it has a structure similar to domain II, including the modified terminal Q-X-W motif (Fig. 2B). Despite the fact that ricin domains have been identified in proteins from bacteria to humans, they are very rare in fungi and absent in S. cerevisiae (http://smart.embl-heidelberg.de /smart/search_keywords.cgi?keywords=ricin). The only other motif identified in the Cup proteins is a putative coiled-coil structure in the C-terminal half. This sequence might be responsible for the weak binding of the Cup proteins to the plasma membrane because truncated CupA, unlike full-length CupB, is completely cytoplasmic in overexpressing cells.

cupA possesses a TGA stop codon near the middle of the gene. This results in two ORFs: one from the initiating ATG to the TGA at positions 1270 to 1272, and a second in a −1 reading frame from just before the TGA to a TAA at a position comparable to the stop codons in the other cup genes. The presence of cDNAs corresponding to cupA in the database (e.g., SLA649) indicates that the gene is transcribed. Moreover, the fact that both reading frames of the mRNA are “open” suggests that they might both be translated in vivo. However, the predicted truncated CupA protein has not yet been detected during Western blot analysis of Ca^{2+}-treated cells (Fig. 3C). This might be due to the poor translation efficiency (or degradation) of the protein (Fig. 3B, C). It is also possible that cupA expression is not up-regulated by Ca^{2+} because cDNAs of this gene were not isolated during SSH. TGA codons within genes are often found at recoding sites involving translational frameshifting (47). A majority of frameshifting events found to date involve −1 frameshifting and occur on a distinctive “slippery heptameric” sequence of the form X-XXY-YYZ (where X = any nucleotide, Y = A or T, and Z = any nucleotide) (17). Certain mRNA secondary structures (e.g., a pseudoknot) just downstream of the slippery heptamer can cause the ribosome to pause and occasionally slip back to a −1 reading frame. In cupA, there are two potential slippery heptamers (A-AAT-TTA) just before the TGA (Fig. 3A), and mfold analysis (http://www.bioinfo.rpi.edu /applications/mfold/old/ma; 55) of the mRNA in this region reveals a putative pseudoknot beginning just 4 nucleotides downstream of the TGA. So far, attempts to detect a full-length CupA due to frameshifting in cells overexpressing the cupA mRNA have been unsuccessful (Fig. 3C). However, this might not be surprising since the translation of cupA mRNA appears to be poor (Fig. 3C) and the efficiency of translational frameshifting is usually low (17). The significance (if any) of this finding to CupA expression is unclear. But, if CupA interacts with the plasma membrane through the C-terminal half of the protein, such a mechanism could produce both membrane-bound and soluble forms.

What is the function(s) of Cup proteins in Dicyostelium cells? Localization experiments indicated that these proteins function in the cytoplasm or are loosely associated with the plasma membrane (Fig. 6). Also, the existence of at least seven cup genes producing very similar proteins suggests that the cells might require large amounts of Cup protein. Therefore, they probably function stoichiometrically rather than catalytically. Since the Cup proteins are quite acidic and their expression is up-regulated by high Ca^{2+} concentration, they could function as Ca^{2+} buffers to help regulate cytosolic [Ca^{2+}] by a mechanism analogous to that of calreticulin in the endoplasmic reticulum (26). However, the primary structure of the Cup proteins shows little similarity to calreticulin and they do not possess known Ca^{2+}-binding structures (e.g., EF hands, C_{2} domains). In addition, pure CupB bound little 45Ca^{2+} in Ca^{2+}overlay experiments (31; B. Coulkell, unpublished data). These results argue against a Ca^{2+} buffer function. But since detection of Ca^{2+} binding by the overlay technique requires SDS-stable, high-affinity binding sites, the possibility of labile, low-affinity Ca^{2+} binding by the Cup proteins cannot be ruled out. Another, more likely, function of the Cup proteins is to stabilize or regulate the plasma membrane during periods of Ca^{2+} stress and/or during certain stages of development. This
idea is supported by several observations. For example, in cells exposed to high Ca\textsuperscript{2+} concentration or undergoing aggregation, Cup proteins become more clearly associated with the cell membrane. In aggregating cells, these proteins appeared to be distributed uniformly around the periphery of the cells (rather than at the front or back) and often displayed a beaded appearance, suggesting local regions of high Cup protein concentration and the degree of suppression appeared to be differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA 93:6025–6030.

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