A Putative Receptor Mediating Cell-density Sensing in *Dictyostelium*

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When *Dictyostelium* cells starve, they begin secreting a glycoprotein called conditioned medium factor (CMF). When there is a high density of starved cells, as indicated by a high concentration of CMF, the cells begin expressing some genes and aggregate using pulses of cAMP as a chemoattractant. CMF regulates gene expression via a G protein-independent pathway, whereas CMF regulates cAMP signal transduction via a G protein-dependent pathway. To elucidate receptors mediating cell density sensing, we used CMF-Sepharose to isolate membrane proteins that bind CMF. We identified a 50-kDa protein, CMFR1, that is sensitive to trypsin treatment of whole cells. We obtained partial amino acid sequence of CMFR1 and isolated the cDNA encoding it. The derived amino acid sequence has no significant similarity to known proteins and has two or three predicted transmembrane domains. Expression of CMFR1 in insect cells caused an increase in CMF binding. Repression of CMFR1 in *Dictyostelium* by gene disruption resulted in a 50% decrease of the CMF binding and a loss of CMF-induced G protein-independent gene expression. The G protein-dependent CMF signal transduction pathways appear to be functional in cmfr1 cells, suggesting that cells sense the density-sensing factor CMF using two or more different receptors.

Cell density sensing mechanisms are used by organisms ranging from bacteria to humans to sense the number of cells of a given type in a region, tissue, or, in the case of the leptin fat composition sensor, the entire body (see Refs. 1–3 for review). Little is known about cell density-sensing signal transduction mechanisms in eukaryotes. The simple eukaryote *Dictyostelium discoideum* uses a cell density-sensing mechanism to coordinate its development. *Dictyostelium* normally exists as vegetative amoebae that eat soil bacteria. When a cell starves, it signals that it is starving by slowly secreting a cell density-sensing factor, the glycoprotein conditioned medium factor (CMF) (4–9). When there is a sufficiently high density of starving cells, as indicated by a high level of CMF, the cells aggregate using relayed pulses of cyclic AMP as the chemoattractant. The aggregated cells then develop into a fruiting body consisting of a mass of spores supported on a column of stalk cells.

CMF has no obvious similarity to any known protein and has no obvious motif content aside from a signal sequence (7). A starved cell secretes roughly 12 molecules of CMF/min and has a CMF sensitivity threshold of 0.3 ng/ml (5). Diffusion calculations showed that CMF can be used for density sensing on a soil surface (8). Interestingly, for aggregates of fewer than ~200 cells, the CMF concentration can never rise to 0.3 ng/ml. These results showed that as a general principle, cells can sense their local density by simultaneously secreting and recognizing a signal molecule. In addition, such a mechanism could be used to determine the total number of cells in a tissue (1, 10, 11).

CMF antisense and knockout cells do not aggregate unless exogenous CMF is added, suggesting that the function of CMF is to coordinate the development of large fruiting bodies by triggering aggregation only when most of the cells in a given area have starved, as signaled by a high level of extracellular CMF (7). Without such a mechanism to sense the density of starved cells, cohorts of cells that starved at the same time might each form a small, ineffective fruiting body.

CMF regulates at least three separate processes in *Dictyostelium*. First, CMF regulates cAMP signal transduction (9). When a pulse of cAMP arrives at a starved *Dictyostelium* cell that is in the presence of high levels of CMF, a burst of cAMP is released by the cell to relay the signal, the cell moves toward the source of CMF, and the expression of specific classes of genes is altered. The incoming cAMP pulse is sensed by cAR1 cell surface cAMP receptors. cAR1s then activate G proteins whose α subunits are Gαs. Gαs normally binds GDP; when activated, Gαs releases GDP and binds GTP, resulting in activation of adenylyl cyclase and guanylyl cyclase (see Refs. 12–16 for review). The GTP is hydrolyzed back to GDP, inactivating the G protein. A CAMP-stimulated Ca2+ influx is mediated partly by a G protein-dependent pathway and partly by a G protein-independent pathway (17, 18). Exposure of cells to CMF is required for CAMP activation of both cyclases and Ca2+ influx (9).

A simple pathway allows CMF to regulate CAMP signal transduction. GTPγS causes a ~40% reduction in the high affinity binding of CMF to membranes, suggesting that some of the putative CMF receptors are coupled to a G protein (19). During the developmental stage when CMF regulates cAMP signal transduction, there is a second G protein present, Go1 (20). Cells lacking Go1 do not exhibit GTPγS inhibition of CMF binding and do not exhibit CMF regulation of cAMP signal transduction, suggesting that a putative CMF receptor interacts with Go1 (19). We found that when CMF binds to this receptor, Gβγ is released from Go1. This activates phospholipase C, which through an unknown mechanism causes the Go2 GTPase to be inhibited, prolonging the lifetime of the
cAMP-activated Gαs-GTP configuration. This, in turn, allows cAR1-mediated cAMP signal transduction to take place (19, 21).

Second, CMF regulates properties of cAR1. CMF binds to developing cells with a $K_0$ of approximately 170 ng/ml (2.1 nm) (6). Vegetative cells have little CMF binding and few cAMP receptors, whereas starved cells possess about 40,000 receptors for CMF and cAMP. Transformants overexpressing cAR1 show a 10-fold increase of cAMP binding and a similar increase of CMF binding; disruption of the cAR1 gene abolishes both cAMP and CMF binding (22). In wild-type cells, high levels of cAMP down-regulate both cAMP and CMF binding, and CMF similarly down-regulates both cAMP and CMF binding. This suggested that cAMP and CMF binding are closely linked. We found that CMF does not bind to cAR1 or cAR2, indicating that the effect of CMF on cAMP binding to cAR1 is through an intermediate (22).2 Binding of $\sim$200 molecules of CMF to starved cells affects the affinity of $\sim$35,000 cAR1s within 2 min, indicating that an amplifying signal transduction mechanism allows one activated CMF receptor to regulate many cAR1s (22). In cells lacking Gβγ, CAMP induced a loss of cAMP binding but not CMF binding, whereas CMF induced a reduction of CMF binding without affecting cAMP binding. These data suggested that CMF regulates properties of cAR1 through a G protein. Two other cAMP-induced events, the formation of a slow dissociating cAMP receptor form and the loss of cAMP binding, also require CMF (22).

The third process regulated by CMF is the expression of prespore- and prestalk-specific genes (4, 5, 7). Expression of these genes requires cells to be exposed to both CMF and cAMP. However, we observed normal CMF/cAMP induction of these genes in cells lacking Gαs, Gαg, Gβγ, phospholipase C, or cytosolic regulator of adenyl cyclase (19). This suggested that, unlike the regulation of cAMP signal transduction and cAR1 properties by CMF, the induction of prestalk and prespore gene expression by the combination of CMF and cAMP is G protein-independent.

Our previous data on CMF binding kinetics did not indicate that there were two classes of CMF receptor with strongly different $K_0$ values (6). Thus there could either be one CMF receptor that mediates both activation of Gαs as well as the G-independent activation of gene expression, or different CMF receptors with similar $K_0$ values, with one activating Gαs and another activating gene expression. There is a precedent for multiple receptors sensing the same ligand in Dictyostelium; pharmacological studies indicate that there are multiple folate receptors (23, 24), and four different cAMP receptors have been cloned, sequenced, and knocked out (25–27). To characterize the receptor(s) mediating cell density sensing, we used an affinity resin to purify a CMF receptor. We found CMFR1, a membrane protein that does not resemble G protein-coupled receptors. CMFR1 appears to mediate the G-independent CMF signal transduction but not the G-dependent CMF signal transduction, suggesting that Dictyostelium cells have two different receptors for CMF.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—Vegetative Dictyostelium** Ax2 and Ax4 cells and mutants were grown in suspension shaking culture with HL5 and antibiotics as described previously (6). To obtain starved cells, mid-log phase cells (2–5 x 10^6 cells/ml) were harvested by centrifugation at 500 x g for 6 min, resuspended in either PB (0.454 g/liter NaHPO_4, 0.945 g/liter KH_2PO_4, pH 6.5) or PBM (20 mM KH_2PO_4, 0.01 mM CaCl_2, 1 mM MgCl_2, pH 6.1 with KOH), pelleted, and resuspended to 1–5 x 10^6 cells/ml at room temperature. Cells were then shaken as for growth conditions for 4–6 h. Developmental stages were followed on filter pads (7), and low density assays for CMF- and cAMP-induced prestalk and prespore gene expression were carried out using immunofluorescence as described previously (28). Conditioned medium was made following Yuen et al. (29).

**Preparation of GST-CMF Fusion Protein and GST-CMF-Sepharose—Recombinant GST-CMF was made and purified following the procedures of Jain and Gomer (6). Typically, 4 liters of bacteria yielded approximately 1–2 mg of fusion protein/0.5 g of resin. GST-CMF-Sepharose was prepared by incubating 0.3 g of glutathione-Sepharose (Amersham Pharmacia Biotech) with freshly thawed bacterial lysate (13 ml) containing approximately 300 μg of recombinant GST-CMF. Con- traction was prepared in parallel by coupling 500 μg of glutathione S-transferase (Sigma) to 0.3 g of glutathione-Sepharose. The coupled resins were then washed with 1 ml of 1% Nonidet P-40/phosphate-buffered saline (80 mM NaHPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) three times and 1 ml of phosphate-buffered saline three times and used immediately for binding preparations.

**Partial Purification of Plasma Membranes and Sequence Analysis of Peptides from GST-CMF-associated Protein—** 6 x 10^6 vegetative or starved cells were pelleted, washed with ice-cold PB, and resuspended in 75 ml of 20 mM NaPB (0.454 g/liter NaHPO_4, 0.945 g/liter NaH_2PO_4, pH 6.5) containing 10% sucrose and Complete protease inhibitor mixture (Roche Molecular Biochemicals), which was present in all purification buffers. Cells were then disrupted at 2 °C using a BioMed-Tekhomogenizer (WVR, Plainfield, NJ) at 150 psi. The particulate fraction was collected by centrifugation at 12,000 x g for 15 min. The pellets containing plasma membranes were resuspended in 6 ml of NaPB/1 mM MgCl_2 and passed 5 times through a 22-gauge needle before bringing the total volume to 15 ml. This fraction was then layered on a step gradient of 7 ml 35% over 2.5 ml 55% sucrose/PB and centrifuged in a Beckman SW41 rotor at 130,000 x g for 60 min at 4 °C following Goeddel-Holland and Luna (30). Membranes were removed from the 35–55% interface, pelleted at 39,000 x g for 15 min, resuspended in 7.5 ml of 0.5% Nonidet P-40/PB, and passed three times through a 22-gauge needle. To solubilize proteins, the sample was then incubated with constant stirring for 1 h at 4 °C, clarified at 43,000 x g for 15 min, and diluted to 45 ml with PB. Nonspecific or GST-binding proteins were removed by first adding 0.3 ml of GST-Sepharose and incubating with constant mixing for 4 h at 4 °C followed by centrifugation to remove the resin. The cleared extract was then transferred to a fresh tube containing 0.3 ml of GST-CMF-Sepharose and incubated with gentle rocking overnight at 4 °C. In some experiments, 1 ml GTPγS, 1 mM GDPβS, or 70 μM cAMP were added during this step. Pelleted resin was then washed in a microcentrifuge tube two times with 1 ml of PB and three times with 0.5% Nonidet P-40/PB, and bound proteins were then eluted with 0.5% Nonidet P-40/PB containing 1 mM NaCl for 1 h at 2 °C (under these conditions the GST-CMF remained tightly bound to the resin). The sample was then separated from pelleted resin by centrifugation and prepared for 15% polyacrylamide gel electrophoresis by desalting and concentrating in 62 mM Tris/HCl (pH 6.8) using a Centricon 10 filter unit (Amicon, Beverly, MA) and stored at −25 °C in Laemmlli’s running buffer (31). This was loaded on a 12.5% polyacrylamide gel that was run with the top chamber containing 40 μl of thioglycolic acid (Sigma) in 200 ml of running buffer. Following electrophoresis, gels were either directly stained with Coomassie R-250, or protein was transferred onto ProBlot (Amersham) PVDF (Applied Biosystems) according to Towbin et al. (32). Selected protein bands were removed for HPLC tryptic peptide or N terminus analyses essentially as described by Jain et al. (7) by the Baylor College of Medicine Protein Chemistry Core Laboratory (Houston, TX).

For whole cell trypsinization studies, 1.4 × 10^8 Ax2 cells were starved for 5 h. The cells were divided into two, collected by centrifugation, then resuspended in either 10 ml of PB or 10 ml PB/10 mg/ml TPCK trypsin (Sigma), and incubated for 15 min at 25 °C. Cells were then washed three times with PB/protease inhibitors, resuspended in 25 ml of 10% sucrose/PB, disrupted in the nebulizer, and processed for GST-CMF binding as described above in a proportionately scaled down manner. GST-CMF-associated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining.

**cDNA Isolation, Cloning, and Sequencing—** The degenerate oligonucleotide primers 5’-TCA/TTGA/TCGA/TT/GCAT/TCA/CAC-3’ and 5’-GCCATCCA/GAA/AA/GAT/CT/TCC/TG/G/A/TA-3’ were synthesized to encode N-terminal and internal tryptic peptide sequences, respectively, of a 50-kDa protein isolated using the above procedure. A YES vegetative Dictyostelium cDNA library was obtained from Dr. Eugeno DeHostos, and PCR was performed using Pfu enzyme (Stratagene, La Jolla, CA) with the above primers. A 1050-bp DNA fragment was gel purified using GeneClean III (Bio 101, Vista, CA), ligated into 2 J. D. Bishop and R. H. Gomer, unpublished data.
PCR 2.1, and cloned using the One Shot E. coli transformation system (Invitrogen, San Diego, CA). Sequencing was then done at the Microbiology Department Core Sequencing Facility (University of Texas Medical School, Houston, TX). Additional sequence was obtained by PCR of the λ YES library using internal oligonucleotide primers 5'-CCCGGCGCATGAGTCCGCAACC-3' and 5'-GCTACTAGTATCTTCAAGAG-3', respectively, and a YES 288lac to gal and 288gal to lac oligonucleotide primers; PCR products were then cloned and sequenced as above. Proteinetics tools on the ExPASy molecular biology World Wide Web server of the Swiss Institute of Bioinformatics were used to predict the pl and hydrophobicity using the algorithms of Eisenberg et al. (33) and hydrophathy by the algorithms of Kyte and Doolittle (34).

Expression of Protein in Insect Cells—The complete open reading frame along with 25 bases of untranslated region upstream of the start codon was obtained for baculovirus expression of CMFR1 by PCR of the λ YES cDNA library described above using the oligonucleotide primers 5'-CCTCAGCACAAATTTGGAAC-3' and 5'-GCTCTAGATCGATCTTCAAGAG-3', which generated 5' EcoRI and 3' XhoI ends, respectively, for cloning into the polyhedrin locus-based transfer vector pVL1392 (PharMingen, San Diego, CA). Following restriction digestions of the PCR product and vector, ligation, and cloning in One Shot E. coli cells, the construct, isolated using a midi Plasmid Kit (Qiagen, Santa Clarita, CA), was identified by the 9800/1500-bp vector/insert fragments of a BamHI/PstI digest. Recombinant baculovirus was generated in SF9 cells (Novagene, Madison, WI) grown in serum-free/antibiotic-free SF-900 II SFM medium (Life Technologies, Inc.). Co-transformation using either 100 ng of Triple Cut Bac-Vector 2000 or 3000 virus DNA (Novagen) and 300 ng of pVL1392 containing the 1540-bp cDNA (above) or control plasmid pVL1392-Xyle (PharMingen) was performed following the Novagen BacVector System Manual. Five days after transformation, aliquots of the medium were used to infect cells for production of high titers of recombinant virus. Optimal infection and expression from the very late polyhrom promoter were determined (after 2–3 days) by following cell cytotoxicity and staining parallel control cells for Xyle E enzyme activity. The recombinant virus construct was partially identified by PCR of PreTag-treated medium samples using the polyhrom promoter primer (Novagen) and cDNA internal primers, followed by sequencing of the PCR product.

Antibody Production and Western Blots—Two synthetic peptides consisting of amino acids 185-202 and 354-369 (see Fig. I) were conjugated to keyhole limpet hemocyanin, and each was then used to immunize a rabbit at Biosynthesis Inc. (Lewisville, TX). IgG was purified from serum using ammonium sulfate (35). Western blots were done following Lindsey et al. (36). For fractionation, SF9 cells were washed with 1% sucrose/2 mM NaCl/PB, resuspended to 109 cells/ml, and frozen and thawed three times. This was then centrifuged at 16,000 × g for 15 min, and the supernatant and pellet were collected and denatured at room temperature for several hours in Laemmli sample buffer (31).

Generation of Gene Disruption Mutants—To generate a gene disruption mutant by homologous recombination, the 1060-bp cDNA-containing plasmid pCMFR1 (see above) was digested at a single internal Sal I site, and the 1060-bp cDNA was ligated into the polyhrom promoter vector, pBluescript SK II (+). The resulting plasmid was transformed into E. coli DH5α, and colonies growing on LB containing 100 μg/ml ampicillin were selected. The 1060-bp BamHI/PstI fragment of the resulting plasmid containing the 9600/1500-bp vector/insert fragments of a SalI internal EcoRI site (57) was digested with EcoRI, gel-purified, ligated to λ YES vector DNA, and used to transfect S. cerevisiae YPH499, which was transformed with linearized λ YES DNA. Transformants were selected on LC plates, and the 1060-bp cDNA fragment described above (100 ng) was labeled with [32P]dCTP by random hexamer-primed DNA synthesis (Amersham Pharmacia Biotech) and used as probe in a Southern blot of genomic DNA prepared using PreTaq (Life Technologies, Inc.) as described by Spann et al. (39).

RESULTS

To identify a possible CMF receptor, we made an affinity resin with recombinant GST-CMF linked to Sepharose. This was incubated with a detergent extract of partially purified plasma membranes from starved Dictyostelium cells. After washing the beads to remove unbound proteins, bound material was eluted with high salt. Several proteins appeared to bind to the GST-CMF resin (Fig. 1). No binding was observed to GST-Sepharose alone (data not shown).

To determine which of these proteins might be a candidate CMF receptor, we tried the assumption that a receptor for CMF would have a domain on the outer side of the plasma membrane. We therefore used tryptic treatment to cleave the whole cells to cleave such proteins. After doing an affinity purification from trypsin-treated and control cells, we saw that a 50-kDa band we named CMFR1 was absent from the material purified from the trypsin-treated cells (Fig. 1C). The other proteins present in the affinity purified material were unaffected by the trypsin

NaCl/PB, respectively, and prepared for 125I-labeled CMF binding by resuspending cells in the respective buffers to a density of 106 cells/ml at 25 °C. Various concentrations of 125I-labeled CMF (0.4–4 nM) with or without a 20-fold excess of unlabeled CMF were prepared in PB with 200 μg/ml bovine serum albumin and clarified for 8 min at 16,000 × g before use. Reactions were initiated by adding 70 μl of the cells to 70 μl aliquots of CMF solution. After 25 min the mixtures were layered on 0.5 ml of sucrose/PB cushions (20% for Dictyostelium cells, 10% for SF9 cells), centrifuged for 2 min at 16,000 × g, and frozen in a dry ice-ethanol bath. Radioactivity was measured by clipping the lower 5-mm region of microcentrifuge tubes into scintillation vials with a canine nail trimmer and counting with Bio-Safe II scintillation mixture (Research Products International, Mount Prospect, IL). All samples were run in duplicate, and a separate duplicate sample of the cell types without radioactivity was taken to quantitate relative cell protein using the Bio-Rad protein assay. Specific cell-associated radioactivity was determined by subtracting values obtained in samples with excess unlabeled CMF (6, 22). Alternatively, reactions were terminated and quantitated by directly pelleting the cell binding samples for 3 min at 16,000 × g, immediately aspirating the solution, and resuspending the pellet with 0.1 ml of 0.1% SDS followed by 1 ml of scintillation fluid. The effect of CMF on cAMP binding to cells in the presence of either ammonium sulfate or PB was measured as described by van Haastert et al. (22), with the exception that cells were starved at 5 × 106 cells/ml for 4 h, and buffer, 2 ng/ml recombinant CMF, or 1:10 Ax4 conditioned medium were added 1 h after starvation. CMF-stimulated IP3 accumulation was measured following Brazill et al. (19).

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FIG. 1. Association of a 50-kDa protein with GST-CMF-Sepharose in crude plasma membrane extracts is destroyed by prior cell trypsinization. Crude plasma membrane fractions from Ax4 cells either not treated (A and B) or treated (C) with 10 ng/ml trypsin/PB were extracted with 0.5% Nonidet P-40 and incubated with GST-CMF-Sepharose in buffer (A and C) or buffer with 70 μM cAMP (B). After washing the resin, the bound proteins were eluted and analyzed by polyacrylamide gel electrophoresis. The arrow points to the 50-kDa CMFR1 protein. The lane at right shows the molecular mass markers.
treatment, indicating that trypsin was not degrading all of the proteins. The degradation by trypsin suggested that CMFR1 might contain a trypsin-sensitive domain present on the outer surface of the plasma membrane. Affinity purification from detergent lysates in the presence of the nonhydrolyzable GTP and GDP analogues GTP$_\gamma$S and GDP$_\beta$S had no effect on the amount of CMFR1 obtained, suggesting that in the lysates CMFR1 does not interact with G proteins, GTP, nor GDP.

To further identify the proteins purified by GST-CMF affinity chromatography, we sequenced the N termini of the different proteins. The N terminus of the 50-kDa CMFR1 protein showed no significant similarity to any known protein (Fig. 2A). The 75- and 53-kDa proteins were identified as DNA K heat shock protein and pyruvate kinase, respectively. The 38-kDa protein was blocked at the N terminus and was not evaluated further. Three peptides from a tryptic digest of CMFR1 were also sequenced and, like the N terminus, showed no similarity to any known protein (Fig. 2A). In conjunction with the previous results, the amino acid sequence data suggested that CMFR1 is a previously uncharacterized membrane protein that binds to CMF.

To determine the complete amino acid sequence of CMFR1, degenerate oligonucleotides corresponding to the N terminus and one of the tryptic fragments were used for PCR, with Dictyostelium cDNA as the template. This produced a 1060-bp product that was cloned and sequenced. Using additional PCR with primers derived from the 1060-bp sequence, we obtained the ends of the cDNA. The assembled sequence showed no significant similarity to any known DNA sequence. The cDNA contained a 1533-bp open reading frame encoding a 510-residue protein (counting from the first methionine) with a predicted mass of 56,497 Da (Fig. 2A). The ATG of the first methionine is preceded by several As, characteristic of many Dictyostelium proteins. A conventional signal sequence begins at residue 36. The N terminus of the isolated protein begins at residue 57, with the predicted sequence from the cDNA match to FIG. 2A, nucleotide and deduced amino acid sequence of cDNA encoding CMFR1. The sequence shown was obtained from cloning PCR products of a vegetative Dictyostelium YES cDNA library. Amino acids 1–56 do not appear to be part of the purified 50-kDa plasma membrane protein. N-terminal amino acid sequence (residues 57–76) from the purified protein is shown with a heavy solid line, and amino acid sequences of tryptic peptide fragments from the purified protein are shown with heavy dashed lines. A putative signal sequence is shown with a light solid line. SalI indicates the site used to insert a blasticidin resistance cassette for a gene disruption construct. B, hydropathy plot of the deduced amino acid sequence. The complete protein sequence (510 amino acids) was analyzed with ProtScale programs based on the algorithm of Kyte and Doolittle (34) with a 9-residue window. The start (amino acid 57) of the 50.5-kDa protein sequence is shown with an arrow. C, output from an algorithm to predict transmembrane domains. The derived amino acid sequence of CMFR1 was analyzed following Hofmann and Stoffel (40). Scores above 500 are considered a significant indicator of a transmembrane domain. The solid line represents the probability that a segment goes from the cytosolic side to the extracellular side; the dashed line indicates the converse.

FIG. 2.—continued
As shown in Fig. 4, the resulting pellet contained intact cells and very little debris. Centrifugation and examined by light microscopy to verify that Insect cells expressing CMFR1 were harvested with gentle CMFR1 protein was present in the particulate fraction of rup-
teative and early developmental stages is consistent with the decrease after 4 h. The presence of the transcript during veg-
ally high affinity cAMP binding observed here is similar to the increase in high affinity CMF binding here is similar to the increase

We expressed the entire cmfr1 cDNA in insect cells. Western blots stained with anti-CMFR1 antibodies indicated that CMFR1 protein was present in the particulate fraction of ruptured cells as a mixture of a 50- and a 56-kDa band (Fig. 4A). Insect cells expressing CMFR1 were harvested with gentle centrifugation and examined by light microscopy to verify that the resulting pellet contained intact cells and very little debris. As shown in Fig. 4B, intact insect cells expressing CMFR1 have a significantly higher level of high affinity CMF binding than cells expressing a control protein. The roughly 4-fold increase in high affinity binding observed here is similar to the increase in high affinity cAMP binding when we expressed the cAR1 cAMP receptor in insect cells. These data indicate that in intact insect cells CMFR1 has an extracellular domain that binds CMF with high affinity.

We further tested the hypothesis that CMFR1 is a CMF receptor, we examined the effect of expressing it in a heterologous system. We expressed the entire cmfr1 cDNA in insect cells. Western blots stained with anti-CMFR1 antibodies indicated that CMFR1 protein was present in the particulate fraction of ruptured cells as a mixture of a 50- and a 56-kDa band (Fig. 4A). Insect cells expressing CMFR1 were harvested with gentle centrifugation and examined by light microscopy to verify that the resulting pellet contained intact cells and very little debris. As shown in Fig. 4B, intact insect cells expressing CMFR1 have a significantly higher level of high affinity CMF binding than cells expressing a control protein. The roughly 4-fold increase in high affinity binding observed here is similar to the increase in high affinity cAMP binding when we expressed the cAR1 cAMP receptor in insect cells. These data indicate that in intact insect cells CMFR1 has an extracellular domain that binds CMF with high affinity.

We previously observed that starved cells bind CMF (6). To determine whether disruption of cmfr1 affects CMF binding to cells, we assayed CMF binding to intact cells. As shown in Table I, disruption of cmfr1 strongly decreases but does not abolish the ability of cells to bind CMF with high affinity.

CMF activates both a G protein-independent as well as a G protein-dependent signal transduction pathway (19). Addition of recombinant CMF and CAMP to starving wild-type cells induces a G protein-independent expression of prestalk and prestalk genes (6, 9, 19, 28). A role of CMFR1 in this pathway was examined by assaying the ability of recombinant CMF and high continuous levels of CAMP to induce prestalk and prespore gene expression at low cell density. We observed a normal induction of gene expression in parental cells, whereas similar treatment of cmfr1 cells failed to induce the expression of these genes (Table I). This suggests that CMFR1 is required for
G protein-independent CMF and cAMP signal transduction.

To determine the role of CMFR1 in G protein-dependent CMF signal transduction, we examined the effect of CMF on cAMP binding and CMF-induced IP$_3$ production. We previously observed that CMF decreases cAMP binding to cells via a pathway that requires the presence of G$_b$ (22). In the presence of ammonium sulfate or PB, cmfr1 cells bind significantly less cAMP than parental cells (Table II). The addition of recombinant CMF decreased cAMP binding proportionately the same in parental and cmfr1 cells (Table II). The addition of recombinant CMF decreased cAMP binding proportionately the same in parental and cmfr1 cells (Table II). To regulate cAMP signal transduction, CMF activates phospholipase C (which produces IP$_3$) via a heterotrimeric G protein (19). We observed that a 30-s stimulus of recombinant CMF increased IP$_3$ levels in both parental and cmfr1 cells (Table II). These data indicate that disruption of cmfr1 does not affect the ability of CMF to modulate cAMP binding or rapidly increase IP$_3$ levels.

**DISCUSSION**

We have partially purified a protein that we hypothesize to be a CMF receptor on the basis of four observations. First, trypsinization experiments show that CMFR1 has a domain on the outer side of the plasma membrane, as observed for eukaryotic receptors of peptide ligands. Second, heterologous cells expressing CMFR1 bind CMF with high affinity. Third, disruption of CMFR1 expression causes a decrease in high affinity CMF binding. Fourth, disruption of CMFR1 abolishes a G protein-independent component of CMF signal transduction.

CMFR1, which shares no significant sequence homology with other proteins, has less than seven hypothetical transmembrane domains and no G protein binding motifs. From Western blots, CMFR1 migrates in SDS-polyacrylamide gels as a ~55-kDa protein, whereas the protein we purified was ~50-kDa, suggesting that there was an alteration in CMFR1 during the purification process. CMFR1 mediates CMF regulation of prestalk and prespore gene expression (Table I), and this regulation occurs via a G protein-independent pathway (19). The above observations suggest that CMFR1 is not coupled to G proteins. CMF regulation of prestalk and prespore gene expression also occurs normally in cells lacking either the R subunit of protein kinase A, Ras, or ERK2 (41–43). Receptors with one (44), two (45, 46), three (47), four (48), and six (49) transmembrane domains have been identified in various systems, and these receptors activate G protein-independent signal transduction pathways. The signal transduction pathway between CMFR1 and prestalk and prespore gene expression thus remains unknown.

Disruption of cmfr1 does not affect the ability of CMF to either alter cAMP binding to cAR1 or to rapidly induce IP$_3$ synthesis. This suggests that CMFR1 does not mediate the CMF regulation of these two events. We previously observed that the regulation of cAMP binding and IP$_3$ synthesis both involve CMF activating a G protein (19, 22). Our data thus corroborate the existence of an unknown G protein-coupled CMF receptor and suggest that that the absence of CMFR1 does not affect this receptor. Interestingly, disruption of cmfr1 does significantly decrease cAR1 levels as indicated by dramatic reductions in cAMP binding (Table II). We previously observed that CMF regulates cAR1 expression (9), so it is therefore possible that CMFR1 plays a major role in cAR1 expression.

The mRNAs encoding both CMF and CMFR1 are present in vegetative and early developing cells and then begin to disappear as the cells aggregate (Ref. 7 and Fig. 3). CMF is sequestered in vegetative cells (29), and we were able to isolate CMFR1 from vegetative cells. Vegetative cells do not secrete CMF and show low levels of CMF binding (22, 29). This suggests that vegetative cells sequester both CMF and CMFR1.

Our results suggest the existence of two CMF receptors in *Dictyostelium*. Scatchard plots of CMF binding to starved cells showed that the binding sites all had the same approximate $K_D$ (6), and we estimate that we would have observed a change in the plot if 10% of the CMF binding sites had a $K_D$ significantly different from 2.1 nM. Disruption of CMFR1 expression decreases CMF binding by approximately 50%. In conjunction with the Scatchard data, this suggests that both CMF receptors have roughly the same $K_D$. In starved cells there are ~40,000 CMF receptors (6), so a first order approximation is that there are 20,000 CMFR1 molecules/cell and 20,000 of the unknown G protein-coupled receptors/cell. With a CMF receptor $K_D$ of 2.1 nM, we previously calculated that an optimal CMF concentration of 1 ng/ml would give ~200 occupied CMF receptors/cell. This would then suggest that 100 occupied CMFR1 receptors

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**TABLE I**

| Binding | A×2 parental cells | cmfr1 cells |
|---------|--------------------|-------------|
| High affinity CMF binding | 100 (def) | 48±7 |
| CP2 | SP70 | CP2 | SP70 |
| Expression marker | - | - | - | - |
| - CMF | ++ | +++ | - | - |

**TABLE II**

| The effect of CMF on cAMP binding and IP$_3$ production in developing parental A×2 and cmfr1 cells |
|-------------------------------------------------|
| A×2 parental cells | cmfr1 cells |
|-------------------|------------|
| cAMP binding, in AMS | 100 (def) | 18 ± 2 |
| cAMP binding, in PB | 100 (def) | 13 ± 2 |
| Ratio of cAMP binding in the presence versus absence of CMF | 0.71 ± 0.08 | 0.72 ± 0.08 |
| CMF-induced increase in IP$_3$ | 1.37 ± 0.22 | 1.48 ± 0.23 |

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3 R. A. Ammann and R. H. Gomer, unpublished data.
regulate prestalk and prespore gene expression and that 100 occupied G protein-coupled receptors regulate cAMP signal transduction and cAR1 properties.

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