Cells Respond to and Bind Countin, a Component of a Multisubunit Cell Number Counting Factor*

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In Dictyostelium discoideum counting factor (CF), a secreted ~450-kDa complex of polypeptides, inhibits group formation and fruiting body size. When the gene encoding countin (a component of CF) was disrupted, cells formed large groups. We find that recombinant countin causes developing cells to form small groups, with an EC_{50} of ~3 ng/ml, and affects cAMP signal transduction in the same manner as semipurified CF. Recombinant countin increases cell motility, decreases cell-cell adhesion, and regulates gene expression in a manner similar to the effect of CF. However, countin does not decrease adhesion or group size to the extent that semipurified CF does. A 1-min exposure of developing cells to countin causes an increase in F-actin polymerization and myosin phosphorylation and a decrease in myosin polymerization, suggesting that countin activates a rapid signal transduction pathway. 125I-Labeled countin has countin bioactivity, and binding experiments suggest that vegetative and developing cells have ~53 cell-surface sites that bind countin with a K_{D} of ~1.5 ng/ml or 60 pm. We hypothesize that countin regulates cell development through the same pathway as CF and that other proteins within the complex may modify the activity of countin and/or have independent size-regulating activities.

The mechanisms multicellular organisms use to form tissues of a genetically predetermined specific size are poorly understood (1, 2). Dictyostelium discoideum is one of the simplest eukaryotic organisms that forms multicellular structures of a defined size (3–8). Dictyostelium normally exists as unicellular amoeboae that eat bacteria on soil surfaces and increase in number by fission. The amoeboae eventually overgrow the bacteria and begin to starve. This starvation triggers a developmental program where the cells first begin secreting conditioned medium factor (CMF),\(^{3}\) a glycoprotein signal (9–16). When there is a high density of starving cells, as indicated by a high concentration of CMF, the cells begin to secrete, sense, and relay pulses of extracellular cAMP as a chemotactant (7, 17–30). Sensing the gradients of the cAMP, the cells aggregate into dendritic streams (31). The aggregated cells form fruiting bodies consisting of a thin, ~1–2-mm high stalk holding up a mass of spore cells. Dispersal of the spores by the wind or insects allows spores to form new colonies of cells.

Although there is selective pressure to have the fruiting body as large as possible for optimal spore dispersal, there is a limit to the mass of spores a stalk can support without toppling or having the spore mass slide down the stalk. Dictyostelium thus regulates the size of fruiting bodies by having the aggregation streams break into groups of up to \(10^5\) cells, with our laboratory strains forming groups of ~2 \(\times\) 10^4 cells on agar (32, 33). We have found that this breakup is mediated by counting factor (CF), a secreted ~450-kDa complex of polypeptides (34). We hypothesized that the levels of extracellular CF allow the cells to sense the number of cells in a local region of the stream and cause breaks in the stream if there are too many cells. smIA^-transformants, which oversecrete CF, form streams that break into a large number of small groups, each of which then forms an abnormally small fruiting body (35). Transformants with a disruption of the gene encoding countin, one of the proteins in the CF complex, form streams that do not break and thus coalesce into abnormally large groups that form huge fruiting bodies that generally topple over. A secreted protein with ~40% identity to countin, countin2, also regulates group size. Instead of causing the formation of smaller groups, countin2 causes the formation of larger groups (36).

Computer simulations indicated that a stream stays intact if the cell-cell adhesion is high and the random cell motility forces are relatively low (37). If the adhesion forces are less than the random motility forces, the cells will instead begin to disperse, disrupting the integrity of the stream. If this dispersal is then followed by a high adhesion and/or low motility, the simulations and observations of cells with altered adhesion indicated that the dispersed cells will then condense into groups, and the size of the groups depends inversely on the extent and length of time the adhesion forces are less than the motility forces (38, 39). We found that CF inhibits cell-cell adhesion by downregulating the expression of known adhesion proteins (37). In addition, CF increases cell motility by increasing the extent of actin polymerization, myosin phosphorylation, and the expression of the ABP-120 actin cross-linking protein, although decreasing myosin polymerization (40).

The expression of adhesion proteins and the extent of actin and myosin polymerization are regulated in part by the relayed pulses of cAMP that mediate aggregation (39, 41–47). We found that a 1-min exposure of cells to CF potentiates the cAMP-induced cAMP pulse, whereas longer exposure of cells to CF inhibits the cAMP-stimulated cGMP pulse (48). CF thus regulates cAMP signal transduction, cell-cell adhesion, the cytoskeleton, and cell motility in order to regulate group size.
The factor that we find regulating group size and the breakdown of a primordium in Dictyostelium is unusual in that it is extremely large and apparently contains five different proteins. In this report we examine the function of one of the component polypeptides, countin. Recombinant countin appears to bind to cells, and within 60 s affects adenyl cyclase and stimulates F-actin polymerization. This suggests that countin or some component of CF that requires countin activates a rapid signal transduction cascade and that the other components may function to modulate the activity of countin and/or may have other activities.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Countin**—A PCR was performed using the primers CCCATGGGACATATGCTGACTATGATATT and CCAGATCTTTAAATGGGACACCTGGA with the Advantage 2 PCR kit (CLONTECH, Palo Alto, CA) and a Dictyostelium developmental cDNA library as the template. This generated a PCR product corresponding to nucleotides 520–1320 of the countin cDNA as numbered in Brock and Gomer (34), with a Ndel site at the 5′ end and a BamHI site at the 3′ end. The purified product was ligated into the Ndel and BamHI sites of pET15b (Novagen, Madison, WI). After sequencing the insert of plasmid pET15b was transformed by the thermal methodology into E. coli BL21 (DE3) (Novagen, Madison, WI), and recombinant countin was expressed following the manufacturer’s directions, with a 5-h induction time. The recombinant countin was purified using a B-PER His 6 Spin Purification Kit (Pierce). Because the protein accumulated as inclusion bodies, these were washed in 20 mM Tris-HCl, pH 7.5, resuspended in 20 mM Tris-HCl, pH 7.5, 4 mM urea, and incubated at 37 °C for 30–60 min. The denatured recombinant countin was then clarified at 20,000 × g for 10 min at room temperature; the supernatant was mixed with the nickel-chelated agarose, and bound protein was purified following the manufacturer’s directions with the exception that 4 mM urea was added to the washing and elution buffers. To refold denatured recombinant countin, a final concentration of 100 μg/ml protein was dialyzed in 20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol at 4 °C for 9 h with three changes of buffer. The protein was then dialyzed in the same buffer without dithiothreitol at 4 °C for 24 h with three changes of buffer. Refolded protein was ready to use after being dialyzed in PBM (20 mM KH2PO4/K2HPO4, pH 6.1, 1 mM MgCl2, 0.01 mM CaCl2) for 24 h with three changes of buffer. The purified protein was quantitated in two ways. First, dilution curves of known amounts of bovine serum albumin and various dilutions of recombinant countin were run on a SDS-polyacrylamide gel and stained with Coomassie Blue R-250, and the lanes were scanned. Second, a Bio-Rad protein assay (Bio-Rad) was performed. The two methods gave similar results. Possible O-glycosylation sites were searched for using the DictyOglyc 1.1 server at www.cbs.dtu.dk/services/DictyOglyc/ (49).

**Biological and Pure Forming Activity of Recombinant Countin**—D. discoideum Ax4 ΔsmlA cells were grown in HL5 media as described previously (34). Cells were harvested at densities of 2–106 cells/ml, 30–μl aliquots of these cells were then spotted onto filters. After 2 h at room temperature, the filters were washed by side on SDS-polyacrylamide gels and stained with Coomassie Blue. AABP04700 filters (Millipore, Bedford, MA) following Brock and Gomer (34), with an Ax4 wild-type, server at www.cbs.dtu.dk/services/DictyOGlyc/ (49). By side on SDS-polyacrylamide gels and stained with Coomassie Blue R-250, and the lanes were scanned. Second, a Bio-Rad protein assay (Bio-Rad) was performed. The two methods gave similar results. Possible O-glycosylation sites were searched for using the DictyOglyc 1.1 server at www.cbs.dtu.dk/services/DictyOglyc/ (49).

**Adenylyl Cyclase and cGMP Assays**—To measure adenylyl cyclase activity, cells were starved in shaking culture in PBM at 1 × 107 cells/ml for 6 h. After washing twice in PB (3 mM NaH2PO4, 7 mM KH2PO4, pH 6.5) in the presence of 2 mM MgSO4, cells were resuspended to 8 × 106 cells/ml and exposed to either 200 ng/ml recombinant countin or buffer for 1 min, and filter-lysed following Parent et al. (28). The basic, unregulated intrinsic, and receptor-mediated adenylyl cyclase activities were measured following Parent et al. (25). cAMP-stimulated cGMP accumulation was measured following Tang et al. (48) with the following modification. Two h after starvation, recombinant countin was added to a final concentration of 200 ng/ml, or an equal volume of buffer was added. Cells were harvested at 6 h and stimulated with 10 mM extracellular cAMP.

**Adhesion, Motility, Actin, and Myosin Polymerization, and Myosin Phosphorylation**—Cell-cell adhesion was measured as described in Roi- sin-Bouffay et al. (37). To measure cell-cell adhesion in vegetative cells, Ax4 cells were grown to log phase (~2 × 108 cells/ml), then diluted to 3–4 × 106 cells/ml in either HL5 or HL5 with 200 ng/ml recombinant countin and allowed to grow in shaking culture. To measure adhesion, a 1-ml aliquot of cells was removed and centrifuged at 340 × g for 2 min, and 750 μl of the supernatant was removed. The cells were resuspended in the remaining 250 μl, vortexed for 5 s, and the sample was rotated on a Labquake rotator (Labindustries, Berkeley, CA) for 2 min. Adhesion assays were performed as described previously (34). One 105 cells/ml, and 200 μl was placed in the well of an 8-well chambered coverslip. 2 μl of 20 μg/ml recombinant countin or an equal volume of buffer was then added to the well. To measure cell motility, cells were videotaped 1 h after being placed in the well, following Yuen et al. (14) with the exception that a 20×20 objective was used. To examine actin polymerization, Ax4 and countin- cells were starved as described above on filters in the presence or absence of recombinant countin. Cells were harvested at 6 h, and preparation of crude cytoskeletons and gel electrophoresis to visualize the level of F-actin was done following Dharmawardhane (52). To determine the effect of treating cells for 1 min with recombinant countin, cells starved in the absence of recombinant countin were harvested, resuspended to 1 × 107 cells/ml in the presence or absence of 200 ng/ml recombinant countin, and stimu- lated with cAMP 80 s later. Myosin polymerization and phosphorylation assays were performed as described previously (40) with the exception that the phosphorylation assays cells were resuspended in the myosin polymerization lysis buffer to 5 × 106 cells/ml, mixed with an equal volume of 2× Laemmli sample buffer, boiled for 3 min, and then electrophoresed on a SDS-polyacrylamide gel. As above, when indicated the cells were treated with 200 ng/ml recombinant countin or an equivalent volume of buffer and were then stimulated with cAMP 80 s later. Gels and autoradiographs were scanned using a phosphorimaging (Molecular Dynamics, Sunnyvale, CA), and peak areas were analyzed using NIH Image (rsb.info.nih.gov/nih-image/). Two-dimensional protein gels and immunofluorescence staining of cells to detect ABP-120 were performed following Tang et al. (40).

**Iodination of Recombinant Countin**—The iodination of recombinant countin was performed by a variation of the chloramine-T oxidation method (13, 53). Recombinant countin was dialyzed against PBK (6.15 mM KH2PO4, 3.85 mM KH2PO4, pH 7.0), and 10 μg in 100 μl was mixed with 6 μl of Na2125I (108 nCi/ml; 17.4 Ci/mg, PerkinElmer Life Sciences) and 20 μl of a freshly prepared solution of 2.5 mg/ml chloramine T (Sigma) in PBK. This reaction was incubated at room temperature for 2 min, and was terminated by the addition of 10 μl of 0.4 mg/ml tyrosine in PBK. Free 125I and 125I-tyrosine were separated from that bound to countin by centrifugation through a Sephadex G-25 (Sigma) column prepared following Sambrook et al. (54) and equilibrated with PBK containing 0.1 mg/ml bovine serum albumin (BSA; New England Bio- labs). The eluate of the column was stored at 80 °C. Aliquots of the reaction were run on a SDS-polyacrylamide gel along with molecular weight standards, stained with Coomassie, and then exposed to Kodak X-OMAT AR x-ray film for 5 days. To quantitate the label, 2 μl of the purified 125I-countin was added to 10 ml of Bio-Safe II liquid scintillant (Research Products International, Mont Prospect, IL) and analyzed in an LS 6500 scintillation counter (Beckman Instruments, Fullerton, CA).

**Binding Assays**—Conditioned medium was prepared from countin-
In group number for both Ax4 and countin concentration showed that there was an increase of a large number of small groups (data not shown). A plot of cells developing in submerged culture caused the formation of a large number of small groups (data not shown).

PBM/BSA were then added, and this was incubated in a water bath at 21°C for 5 min at room temperature. 50 μl of HB9262 was incubated for 5 min in PBM/10 μg/ml BSA. A mixture of 10 μl of countin CM or PBM and 10 μl of various amounts of 125I-countin (and unlabeled countin where indicated) in PBE was incubated for 5 min at room temperature. 50 μl of cells or PBM/BSA were then added, and this was incubated in a water bath at 21°C for 10 min, unless specified differently. The 70-μl reaction was then carefully layered on a 0.5-ml cushion of 20% sucrose, 10 μg/ml BSA, 100 mM PB in an Eppendorf centrifuge tube, centrifuged at 160 × g for 2 min, and then quickly frozen in an ethanol/dry ice bath. The bottom of the frozen tube (containing the cell pellet) was cut off with a veterinary nail clipper and placed in a scintillation vial with 0.5 ml of distilled water. After thawing, 10 ml of scintillation fluid was added to the vial; the vial was vortexed again, and the radioactivity of each sample was counted twice for 5 min.

**RESULTS**

**An ~50-kDa Dimer of Recombinant Countin Has the Bioactivity of the 450-kDa CF—**We found previously (34) that disrupting the expression of countin, one of the components of CF, essentially abolishes CF activity. This indicated that either countin was a key component of CF that directly affects cells or that countin was simply a necessary part of the CF. To begin to distinguish between these two possibilities, we determined whether countin alone has any of the regulatory properties of CF. We expressed the countin polypeptide backbone in bacteria and found that it could be purified to a 27-kDa band that stained with anti-countin antibodies (Fig. 1). This molecular weight is what was predicted from the derived amino acid sequence. When recombinant countin was added to cells starved on filters, it increased the number of groups and decreased the number of cells per groups for both Ax4 and countin− cells (Fig. 2A). Similarly, addition of recombinant countin to cells developing in submerged culture caused the formation of a large number of small groups (data not shown). A plot of the number of groups of cells formed as a function of recombinant countin concentration showed that there was an increase in group number for both Ax4 and countin− cells up to a concentration of 100 ng/ml (Fig. 2B). At 1000 ng/ml of recombinant countin, there was less of an effect compared with that seen with 100 ng/ml, suggesting the presence of a peak in the dose-response curve. The EC50 of recombinant countin was ~3 ng/ml using Ax4 cells and ~11 ng/ml using countin− cells. Together, the data suggest that recombinant countin can affect group size in developing Dictyostelium cells.

The sequence of countin has a motif similar to that of amoebapore, polypeptides that are secreted by the pathogenic amoeba E. histolytica (55). These polypeptides form pores in target cell membranes (50, 51). Recombinant countin was thus assayed for pore-forming activity. Compared with 1200 units/ng for amoebapore A, countin showed no significant activity at pH 8.0, 7.7, 7.0, 6.5, and 6.0, whereas at pH 5.5 countin had 8.6 ± 0.7 units/ng, and at pH 5.2 it had 8.3 ± 0.8 units/ng (means ± S.D. from 3 independent assays). The data indicate that at low pH countin has a small but measurable membrane-perturbing/destabilizing activity, suggesting that below pH 6.0 countin interacts with phospholipids and membranes (56, 57).

However, countin does not appear to display a true pore-forming activity as amoebapores (58), in particular at pH values between 6 and 7, the typical pH values at which Dictyostelium cells grow and develop.

Because the CF preparation contains multiple polypeptides, one possibility is that the CF activity is because of a 450-kDa polymer of countin, and the other proteins are simply contaminants. To determine whether recombinant countin functions as a monomer or as a multimer, we performed liquid chromatography of recombinant countin on a size-exclusion column and measured the bioactivity of each fraction. We found that the activity that caused Ax4 cells to form large groups eluted at ~60 kDa. Ultracentrifugation of recombinant countin solutions indicated that recombinant countin behaves in solution as a 47.3-kDa molecule. Together, the data suggest that recombinant countin forms a dimer.

**Like CF, Recombinant Countin Decreases Cell-Cell Adhesion and Increases Cell Motility—**Semi-purified CF causes a reduction of cell-cell adhesion (37). To determine whether recombinant countin also has this property, we starved cells in the presence or absence of recombinant countin and measured cell-cell adhesion. Exposure of cells to 0.2 μg/ml recombinant countin for 2 h decreased cell-cell adhesion (Fig. 3A). At 4 h of development, the recombinant countin decreased adhesion by ~13%; this is roughly comparable with the 20% decrease caused by 0.1 μg/ml (or the ~10% decrease caused by 0.05 μg/ml of semi-purified CF (37)).

smlA+, wild-type, and countin− cells have different levels of adhesion immediately after cells have starved (37). To determine whether recombinant countin regulates cell-cell adhesion in vegetative cells, Ax4 cells were harvested and resuspended in HL5 with or without 200 ng/ml recombinant countin. At 2, 4, and 6 h after treatment with recombinant countin, cells were collected, and cell-cell adhesion was measured. As shown in Fig. 3B, recombinant countin inhibited cell-cell adhesion in vegetative cells. The decreasing adhesion observed as a function of time in the vegetative cells appears to be a result of harvesting the cells. Observations on cells that had not been centrifuged showed that their adhesion was constant over time (data not shown). To determine whether countin quickly regulates cell-cell adhesion, cells were treated with recombinant countin for 1 min, and their adhesion was then measured. By taking into account the time needed to centrifuge the cells and the 2-min incubation, we found that recombinant countin decreased cell-cell adhesion within 4 min in vegetative cells. (Fig. 3B).

In addition to regulating adhesion, CF potentiates cell motility (40). When cells were exposed to recombinant countin, we observed an increase in motility (Fig. 4). The degree of in-
creased motility was essentially indistinguishable from the increased motility observed in \textit{smlA} cells, which oversecrete the entire CF complex (see Ref. 40, and data not shown). Together, the data suggest that recombinant countin can substitute for CF to decrease cell-cell adhesion and increase cell motility.

Recombinant Countin Affects Actin and Myosin within 60 s—

The CF complex appears to regulate motility and group size in part by regulating actin polymerization (40). To determine whether recombinant countin also has this activity, cells developing on filters were starved and then transferred to pads soaked with buffer or 0.2 \mu g/ml recombinant countin. The cells were then harvested and stimulated with cAMP. A Coomassie-stained gel of crude cytoskeletons showed two characteristic peaks of F-actin in wild-type cells (52, 59, 60). When these cells had been exposed to recombinant countin, the level of polymerized F-actin increased, but no definite peak appeared (data not shown), a pattern that we previously observed in \textit{smlA} cells (40). As observed previously (40), there was only one peak of F-actin evident in \textit{countin}\textsuperscript{−} cells (Fig. 5, A and B). When these cells were exposed to recombinant countin, a second peak of F-actin appeared, mimicking the time course observed in wild-type cells (52, 59, 60). This indicated that recombinant countin not only increases F-actin polymerization but also affects the time course of F-actin stimulation by cAMP. To determine how long it takes for recombinant countin to affect the cAMP stimulation of actin polymerization, developing cells were harvested after 6 h of starvation, exposed to 0.2 \mu g/ml recombinant countin for 60 s, and then stimulated with cAMP. A strong potentiation of the second peak actin polymerization was observed, suggesting that cells can sense recombinant countin and regulate the cAMP-to-actin pathway within 1 min (Fig. 5, A and B). Although F-actin levels were significantly altered when cells were exposed to recombinant countin for 60 s, the same treatment failed to alter cell motility. We found previously (40) that CF increases the levels of ABP-120. Both two-dimensional gels and immunofluorescence of cells stained with anti-ABP-120 antibodies showed that exposure of starving wild-type cells to 200 ng/ml recombinant countin for 4 h increased the levels of ABP-120 (data not shown). Together, the data indicate that recombinant countin has the same effect as CF on the expression of ABP-120 and actin polymerization.

CF also increases myosin phosphorylation and decreases myosin polymerization (40). A cAMP pulse causes a transient increase in myosin polymerization (61–64). This transient increase in myosin heavy chain phosphorylation occurs on threonine residues and causes myosin to depolymerize at the leading edge of the cell (41, 65–73). A 1-min exposure of cells to recombinant countin increased the basal level of myosin phosphorylation (Fig. 5, C and D), although there was no effect of this exposure on the total levels of myosin heavy chain (Fig. 5G). A 1-min exposure of cells to recombinant countin decreased the level of polymerized myosin heavy chain (Fig. 5, E and F) and increased the levels of soluble myosin (data not shown). Exposure of cells for several hours to recombinant countin also increased the basal levels of myosin heavy chain phosphorylation and decreased the levels of myosin heavy chain.
chain polymerization (data not shown). The above data indicate that within 1 min recombinant contin has the same effect as long term exposure of cells to CF or recombinant contin on myosin phosphorylation and polymerization.

Recombinant Contin Affects Adenylyl Cyclase Activity within 60 s—We showed previously (48) that CF regulates the cAMP-induced cAMP pulse size. To determine where in the pathway CF regulates the cAMP pulse, we examined the effect of recombinant contin on adenylyl cyclase. Cells were exposed to 200 ng/ml recombinant contin for 1 min before the measurement of adenylyl cyclase activity. Exposure of cells to recombinant contin did not affect the basal or unregulated in-

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**Fig. 3.** Recombinant contin decreases cell-cell adhesion. A, Ax4 cells were starved in the presence or absence of 0.2 μg/ml recombinant contin. Values are means ± S.D. from three independent experiments. B, Ax4 cells growing in shaking culture were incubated with or without 200 ng/ml recombinant contin for the indicated times, and adhesion of the vegetative cells was measured, using criteria that are more sensitive for low levels of adhesion than the assays used for the data shown in A. Values are means ± S.E. from three separate experiments.
trinsic adenyl cyclase activities (Fig. 6). However, a 60-s exposure to recombinant countin increased the GTPγS-stimulated activity (Fig. 6). We also found previously (48) that CF inhibits guanylyl cyclase, thus reducing the cAMP-stimulated cGMP pulse size, but that this effect requires cells to be exposed to CF for several hours. To determine whether countin itself is capable of regulating the cGMP pulse size, recombinant countin was applied to cells 2 h after starvation, and the cAMP-induced cGMP pulse was measured at 6 h after starvation. As shown in Fig. 7, recombinant countin decreased the cAMP-stimulated cGMP pulse size. This suggests that CF regulates the size of the cAMP-stimulated cGMP pulse at least partially through its countin subunit.

Development of a Binding Assay—To determine whether countin is sensed by cell-surface receptors, we examined the binding of countin to intact cells. After iodination, we found that the molar ratio of countin protein to incorporated 125I was 1:1.6. Fig. 8 is an autoradiogram of the iodinated protein electrophoresed on a SDS-polyacrylamide gel that was stopped and immediately exposed to film when the dye front was 1.5 cm from the bottom of the gel. On these gels free 125I and 125I-tyrosine run at the dye front. There was no detectable degradation of the protein and very little free 125I or 125I-tyrosine present after centrifugation over the Sephadex G-25 column. To check if the iodination reaction caused any major damage to the activity of recombinant countin, the proteins were assayed for CF activity by developing cells in submerged culture in the presence of different concentrations of 125I-labeled or unlabeled countin. The EC_{50} of 125I-countin was roughly 20 ng/ml compared with ~3 ng/ml for unlabeled countin. This suggested that the 125I-countin retained bioactivity but that the iodination reduced the potency.

The time course of 125I-countin binding was first examined to establish steady state conditions to carry out more complex binding assays. For this, countin- cells were starved for 6 h at a density of 1 × 10^6 cells/ml. To examine the kinetics, the cells were allowed to bind to a fixed concentration of 125I-countin for varying amounts of time. Fig. 9 shows that the 125I-countin bound to intact developing cells rapidly with the maximal binding being reached by 10 min. This level of binding remained relatively constant for 30 min. When the 125I-labeled countin was preincubated with conditioned medium from countin- cells (to allow countin to bind to components of CF that may be secreted by countin- cells), there was a somewhat higher level of binding. The binding was not significantly affected by the addition of 2 μM dithiothreitol, 0.05% Nonidet P-40, or 3% glycerol to the binding reaction and/or cushion or changes in the cushion buffer composition, suggesting that the binding was somewhat robust.

Dictyostelium Cells Have High Affinity Countin-binding Sites—The minimal requirement for binding to be considered significant is that it should be saturable. To examine this, cells at 6 h of development were incubated with varying concentrations of 125I-countin, and the bound radioactivity was determined after 30 min of incubation (Fig. 10A). The binding appeared to saturate with a maximum number of binding sites of roughly 53 per cell in the absence of exogenous CM. When countin- CM was added to the binding reaction, the binding partially saturated at roughly 27 sites per cell, but the binding increased at higher 125I-countin concentrations, suggesting that the presence of the CM caused some amount of nonspecific binding. The average dissociation constant, K_{D}, for 125I-countin appears to be ~10 ng/ml or ~0.4 nM in the absence of exogenous CM and ~5 ng/ml or ~0.2 nM in the presence of exogenous CM. The Hill coefficient calculated from the steady state binding is 2.3, which indicates that there is an apparent cooperativity between the binding sites. To determine the K_{D} for the binding of unlabeled countin, we measured the binding of a fixed amount of 125I-countin in the presence of varying amounts of unlabeled countin. As shown in Fig. 10B, in the absence of exogenous CM the binding was competed until the unlabeled countin concentration reached ~5 ng/ml, and then roughly plateaued. This plateau value represents the nonspecific binding (74). The approximate concentration where half of the specific binding is competed for by the unlabeled countin is ~1.5 ng/ml, indicating that the K_{D} value for binding of unlabeled recombinant countin to cells is ~1.5 ng/ml. Because this is less than the K_{D} value for binding of 125I-countin to cells, the data suggest that the iodination of countin somewhat reduces its ability to bind to cells.

To determine when during development the countin binding activity was present, binding was measured at 0, 2, 4, and 6 h after starvation. As shown in Fig. 11, high affinity binding can be detected throughout early development. There was no ap-

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FIG. 4. Recombinant countin increases cell motility. Cells were starved in the presence or absence of 200 ng/ml recombinant countin, and the speed of cell movement was measured at 6 h. The average speed of Aax cells exposed to PBM buffer was 5.8 ± 0.4 μm/min, whereas for recombinant countin it was 9.9 ± 0.6 μm/min (means ± S.E.; p < 0.001 using a Mann-Whitney Rank Sum test).
parent change in the number of binding sites per cell or the apparent $K_d$ value of the binding (data not shown). Together, the data suggest that growing and developing cells have high affinity binding sites for countin.

**DISCUSSION**

**Countin Has Some CF Activity**—We have found here that the bacterially synthesized polypeptide backbone of countin appears to have the biological activity of the entire complex. The predicted amino acid sequence of countin contains potential N-linked glycosylation sites (34), as well as potential O-linked glycosylation sites at amino acids 181 and 204. Because native countin is 40 kDa and the backbone is 27 kDa, our data suggest that the posttranslational modifications of countin (presumably glycosylation) are not necessary for its activity. All of the work presented here was done with cells secreting the other components of CF, so we do not know if recombinant countin can function without these components of CF. Cells with a disruption of the gene encoding CF50 (another component of CF) form large fruiting bodies (75). We observed that recombinant countin affects group size in cfs0 cells, suggesting that CF50 is not necessary for the activity of countin. The optimal concentration of purified CF caused an ~200% increase in group number and a 20% decrease in adhesion (34, 37), whereas recombinant countin caused an ~89% increase in group number and a 13% decrease in adhesion. The observation that at or near its optimal concentration recombinant countin does not cause as great an increase in group number or as great a percent decrease in adhesion as purified CF suggests that the other components of CF are needed for a maximal change in group number. Thus one possibility is that other components of CF separately affect group number.

**Countin Activates a Rapid Signal Transduction Pathway**—CF potentiates the cAMP-stimulated cAMP pulse without affecting the kinetics of the cAMP receptor, cAMP-induced GTP binding to membranes, the subsequent GTP hydrolysis, the GTPase inhibition of cAMP binding, or the binding of the cytosolic regulator of adenyl cyclase (CRAC) to membranes (48). The binding of CRAC to membranes is due to cAMP activating a phosphatidylinositol 3-kinase, which creates phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate on the inner surface of the plasma membrane; a

The heavy band visible in the figure is at 42 kDa and was previously identified as being actin (52). B, gels from three independent experiments performed as in A above were scanned, and the average actin binding densities were plotted. C, Ax4 cells were starved, exposed to recombinant countin and cAMP as in A, and at the times indicated after cAMP stimulation the cells were solubilized in Laemmli sample buffer and electrophoresed on a SDS-polyacrylamide gel. A Western blot of the gel was stained with anti-phosphothreonine antibodies; the band visible at 42 kDa is the phosphorylated myosin heavy chain (40). D, autoradiograms from two independent experiments as in C were scanned, and the average phosphorylated myosin band intensities were plotted. E, cells were starved for 6 h, collected, exposed to recombinant countin or buffer for 1 min, and then were stimulated with cAMP as in A; crude cytoskeletons containing myosin were prepared at the times indicated after stimulation. The cytoskeletons were electrophoresed and stained as above; the heavy band visible is myosin heavy chain. F, gels from two independent experiments as in E were scanned, and the average myosin band intensities were plotted. G, the membrane used for the Western blot in C was stripped and rewarmed with anti-myosin antibodies.

**Fig. 5.** A 1-min treatment of countin− cells with recombinant countin increases actin polymerization and myosin phosphorylation and decreases myosin heavy chain polymerization. A, ccountin− cells were starved for 6 h in shaking culture, exposed to 0.2 μg/ml recombinant countin for 1 min, and then stimulated with cAMP. Samples were taken at the indicated times in seconds after the cAMP stimulation and subjected to a rapid extraction to obtain crude cytoskeletons containing actin. These were then electrophoresed on an SDS-polyacrylamide gel, and the proteins were visualized with Coomassie. parent change in the number of binding sites per cell or the apparent $K_d$ value of the binding (data not shown). Together, the data suggest that growing and developing cells have high affinity binding sites for countin.
pleckstrin homology domain on CRAC then binds to these lipids (28, 76). We find that recombinant countin modulates the GTP\(_7\)S-stimulated activity of adenylyl cyclase without affecting the basal or Mn\(^{2+}\)-stimulated activities. Assuming that countin affects the same pathway as CF, this suggests that CF affects the cAMP-stimulated cAMP pulse at a step between the binding of CRAC to membranes and adenylyl cyclase.

We previously observed that purified CF potentiated the cAMP-stimulated cAMP pulse within 60 s. We find here that a 60-s exposure of cells to countin can decrease myosin polymerization and increase actin polymerization, myosin phosphorylation, and the GTP\(_7\)S-stimulated activity of adenylyl cyclase. This suggests that countin, like CF, stimulates a rapid signal transduction pathway that has a direct effect on actin polymerization and a modulating effect on the cAMP receptor to adenylyl cyclase pathway.

There Are a Small Number of Countin Receptors—There are about \(4 \times 10^4\) receptors on \(D\)ictyostelium cells for the glycoprotein cell density sensing factor CMF and an equal number of receptors for the chemoattractant cAMP (13, 22, 77). However, we find that there appears to be only \(\sim 53\) receptors for countin. This low number of receptors is not entirely unusual. Jurkat cells have \(-140\) somatostatin receptors (78); human monocytes have \(-22\) interleukin-2 receptors per cell (79), and human eosinophils have \(-200\) receptors for interferon-\(\gamma\) (80). Human urothelial cells have \(-40-100\) interferon-\(\alpha\)2B receptors per cell (81). For the glycoprotein granulocyte-macrophage colony-stimulating factor, there are \(-74\) receptors on HL-60 cells, and even lower numbers of receptors on other granulocyte-macrophage colony-stimulating factor-sensitive cells, with as low as \(8\) receptors per cell on human monocytes (82, 83). There exist mouse interleukin-6-dependent plasmacytomas with \(10-15\) receptors per cell (84).

We calculated previously (34) that wild-type cells secrete \(~60\) molecules of CF per cell per min. If we assume that cells secrete CF at an even rate, then, for instance, the actin polymerization experiment where cells were collected by centrifugation, resuspended, and treated with recombinant countin for
60 s, the cells could accumulate \(6 \times 10^8\) molecules/ml of CF, or \(1 \times 10^{-13}\) M CF. Because the EC\(_{50}\) for recombinant countin is 3 ng/ml or \(1 \times 10^{-10}\) M (and we were actually using 100 ng/ml recombinant countin), this suggests that there would not have been enough of the other components of CF to form a significant amount of CF complexes with the recombinant countin. This then suggests that countin can interact directly with cells to activate a signal transduction pathway.

Other Components of CF also Affect Group Size—Our data on the number of groups formed as a function of recombinant countin concentration indicate that the EC\(_{50}\) for recombinant countin is 3 ng/ml. Our binding data suggest that the KD for recombinant countin binding is \(1.5\) ng/ml. By using \(B = RTK_D^2/F + 1\), where \(B\) is the number of occupied receptors per cell, \(RT\) is the total number of receptors per cell, and \(F\) is the free concentration, we find that at 3 ng/ml recombinant countin, \(-66\%\) of the countin receptors are occupied. The EC\(_{50}\) for CF is roughly 100 ng/ml (34). Assuming that countin is 80/450 = 17% of CF, then at this concentration of CF there is \(-17\) ng/ml countin. This suggests that the EC\(_{50}\) for recombinant countin is lower than the EC\(_{50}\) for countin when it is part of CF. We observed that when countin- CM is added to the binding reactions, the binding of \(125\)I-countin decreases somewhat at low \(125\)I-countin concentrations and increases at high \(125\)I-countin concentrations. This suggests that some component in the CM interacts with the \(125\)I-countin and modifies its binding to cells. In conjunction with the observation that recombinant countin does not have as large an effect on group number as purified CF, the above results indicate that the other components of CF, such as CF50 (75), may modulate the activity of countin and that, together with countin, they may allow CF to modulate group size.

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**Fig. 8. Iodination of recombinant countin.** Recombinant countin was labeled with \(125\)I using chloramine T. The purified labeled protein was electrophoresed on a 12.5% SDS-polyacrylamide gel, and the gel was exposed to x-ray film for 15 min. Numbers on the left are positions of molecular mass markers in kDa.

**Fig. 9. Time course of countin binding.** countin- cells were starved for 6 h, washed, and incubated with 52 ng/ml \(125\)I-countin for 1, 3, 10, or 30 min in the presence or absence of conditioned medium. Bound values were obtained by subtracting the no-cell counts from counts with cells. Values are means \(\pm\) S.E. from three separate experiments.

**Fig. 10. The effect of different amounts of \(125\)I-countin or unlabeled countin on binding.** A, countin- cells were starved for 6 h, washed, and incubated with increasing amounts of \(125\)I-countin for 10 min in the presence or absence of countin- conditioned medium. Bound values were determined by subtracting the counts from control experiments done without cells from those with cells. Values shown are means \(\pm\) S.E. from 14 separate experiments. B, cells starved for 6 h were incubated with 8 ng/ml \(125\)I-countin and the indicated amounts of unlabeled recombinant countin. Values are means \(\pm\) S.E. from eight separate experiments.

**Fig. 11. Countin binding during development.** countin- cells were starved for 0, 2, 4, or 6 h and resuspended to a density of \(2 \times 10^7\) cells/ml. The cells were incubated with increasing amounts of \(125\)I-countin as in Fig. 10A. The approximate amount of high affinity saturable binding sites was then calculated for each time point. Values are means \(\pm\) S.E. from three separate experiments.