A Cytosolic Cyclic AMP-dependent Protein Kinase in Dictyostelium discoideum

II. DEVELOPMENTAL REGULATION*

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The CAMP-dependent protein kinase of the cellular slime mold, Dictyostelium discoideum, is developmentally regulated; there is an approximately 4-fold increase in activity during development. The incorporation of [3H]leucine into the enzyme demonstrates that there is de novo synthesis of the CAMP-dependent protein kinase. The activities of the catalytic and regulatory subunits increase in parallel. The maximal rate of increase of CAMP-dependent protein kinase activity precedes "tip" formation, a stage of development characterized by a sharp increase in mRNA complexity. The high level of CAMP-dependent protein kinase activity, attained at this stage of development, persists when aggregates are dispersed and the amoebae are kept in suspension without added CAMP. The synthesis of the developmentally regulated mRNAs under these conditions is dependent on exogenous CAMP. The increase in CAMP-dependent protein kinase activity during development does not require sustained cell-cell contact insofar as it occurs in single cell suspensions of amoebae. Furthermore, the increase does not require exogenous CAMP, although added CAMP stimulates the synthesis of the enzyme to a level higher than that found, when CAMP is not added. These observations support the hypothesis that in Dictyostelium discoideum CAMP-dependent protein kinase mediates the effects of CAMP on development.

The advantages of the cellular slime mold, Dictyostelium discoideum, as an object for the study of cellular differentiation and morphogenesis have been discussed at length (1, 2). The CAMP-dependent protein kinase of the organism, such as ease of obtaining large quantities of amoebae, synchronous with respect to stage of development, availability of developmental mutants, and the recent isolation of clones coding for mRNA characteristic of cell type and stage of development (3, 4), Dictyostelium discoideum is of particular interest insofar as it represents the deepest known branch of eukaryotic phylogeny (5) and insights gained from its study may well be of wide applicability. A function of CAMP in the overall development and morphogenesis of the organism is well established and a role in cellular differentiation seems likely, if as yet unproven. The CAMP-dependent protein kinase of D. discoideum is a potential mediator of the effects of CAMP. In the preceding paper we characterized the enzyme. We demonstrated that, contrary to an earlier report (6), CAMP-dependent protein kinase occurs in vegetative as well as in developing amoebae. If CAMP-dependent protein kinase were indeed to mediate the effects of CAMP, then one would expect different levels of enzyme activity at different stages of development. While changes in cellular levels of CAMP suffice to bring about changes in CAMP-dependent protein kinase activity, changes in the amount of the enzyme might also occur. The observations described in the present paper show that this is indeed the case. Furthermore, other workers found earlier that, when aggregates of amoebae were dispersed at the "tip" stage of development, the synthesis of certain developmentally regulated mRNAs and proteins in single cell populations ceased. The addition of CAMP to such suspensions prevented the cessation of the synthesis of at least some of these mRNAs and even brought about their resynthesis, if added after their disappearance (3, 4, 7). We find that the developmentally regulated CAMP-dependent protein kinase is metabolically stable when the aggregates are dispersed and the amoebae are incubated without exogenous CAMP.

Taken in toto, our findings provide strong evidence for a role of the CAMP-dependent protein kinase in mediating the effects of CAMP on the development of Dictyostelium discoideum.

EXPERIMENTAL PROCEDURES

Strain and Conditions of Growth and Development—D. discoideum, strain AX3/RC3, was grown to a density of 2–4 × 10⁶ amoebae/ml on Medium HL5, as described previously (8). The amoebae were harvested by sedimentation, washed twice in PDF buffer (9) (50 mM potassium phosphate, pH 6.5; 20 mM potassium chloride; 2.5 mM magnesium chloride) and processed immediately when vegetative amoebae were required or permitted to develop either on PDF agar (9) at a density of about 6.5 × 10⁶ amoebae/cm² in the dark at 22 °C or incubated at a density of 1 × 10⁶ amoebae/ml in PDF buffer with shaking at 200 rpm at 32 °C.

Preparation of Extracts.—The amoebae were either scraped from agar plates into PDF buffer or sedimented from suspension; all subsequent operations were performed at 0–4 °C. Amoebae were washed once in 20 mM MOPS, pH 7.2; 1 mM EDTA (ME), then suspended to a density of about 3–4 × 10⁶ amoebae/ml in lysis buffer.

The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; 8NcAMP, 8-azido adenosine 3′:5′-monophosphate; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Rn, regulatory subunit of CAMP-dependent protein kinase isozyme II.

† This research was supported by a grant from the American Cancer Society (ACS-BC-375). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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chymostatin, leupeptin, pepstatin, and soybean trypsin inhibitor and immediately disrupted by sonication for 10 s at a setting of 1 in a sonic cell disruptor (Heath Systems Ultrasone). Debris was removed by centrifugation for 15 min at 30,000 × g and the supernatant fluid was used for detection of the regulatory subunit (after passage of an aliquot through a Sephadex G-25 column) and of the catalytic subunit (after passage through a column of chromafocusing support PBE 94).

**Quantitation of the Regulatory Subunit of the cAMP-dependent Protein Kinase**—An aliquot of the supernatant fluid was processed through a column (0.5 × 15 cm) of Sephadex G-25, washed with 20 mM Tris-HCl, pH 7.0, 1 mM EDTA, 10% glycerol, 0.1 mM benzamidine, and 1 mM PMSF. The fractions containing the bulk of protein (detected visually by the Bio-Rad protein assay) were pooled. We found in control experiments that less than 0.1% of added cAMP was in the effluent, when the nucleotide was added at a concentration of 100 μM to the material applied to the column. The protein fraction was photoaffinity-labeled in triplicate at 0 °C with 8N-[^32P]cAMP (200 nM) in 25 mM MOPS, pH 7.0, 4 mM MgCl₂, 0.4 mM EGTA, and 5 mM NaF. Exposure to ultraviolet light from a UVS 11 source was for 1 min at a distance of 2 cm and was initiated 30 s after the protein and the photoaffinity probe were mixed. The sample was then boiled in concentrated SDS electrophoresis buffer and subjected to one-dimensional gel electrophoresis (10) and autoradiography (11). The band corresponding to the regulatory subunit was excised and the radioactivity was determined. Regulatorubunit subunit activity is expressed as femtomoles of 8N-[^32P]cAMP covalently bound per mg of protein in the pooled protein fraction.

**Quantitation of Catalytic Subunit**—An aliquot of the 30,000 × g supernatant fraction was diluted 1:1 with 25 mM imidazole-HCl buffer, pH 7.4, 1 mM EDTA, 2 mM mercaptoethanol, 10% glycerol, and 0.5 mM PMSF and layered onto a column (0.5 × 10.5 cm) of PBE 94 chromatofocusing gel pre-equilibrated to pH 7.4 with the same imidazole-HCl buffer. The column was eluted with Polybuffer 74, pH 4.5, 2.5 mM MgCl₂, according to manufacturer’s instructions and supplemented with 1 mM EDTA, 2 mM mercaptoethanol, 10% glycerol, and 0.5 mM PMSF. Alternate fractions were used for the determination of pH and of phosphorylating activity under three conditions. Phosphorylating activity was measured in 25 mM MOPS, pH 7.0, 4 mM MgCl₂, 2 mM dithiothreitol, 5 mM NaF, and 100 μM [γ-[^32P]ATP at either 0.5 μM M, 1 μM M, 10 μM M, or 1 μM M of cAMP or with 3 μg/ml of casein (pretreated, as described by Reimann et al. (12)) and 1 μM M, as the respective substrates. Samples were processed after 15 min of incubation at 30 °C as described earlier (8). Activity is expressed as picomoles of phosphorylated substrate formed per min. The cAMP-inhibited rat histone kinase was assayed as described by Langan (13).

**Disaggregation of Aggregates**—Amoebae were allowed to develop on agar for 12–14 h (see Fig. 3 for stage of development); they were then scraped from the plates into PDF buffer, washed in the same buffer, suspended in PDF buffer containing 10 mM EDTA, and vortexed for 5 min. Aliquots at a density of 1 × 10⁶ amoebae/ml of suspension were incubated with shaking at 200 rpm for 5–6 h in either the presence or absence of 1 mM cAMP added at 0 h and at 2 and 4 h after disaggregation; 350 μg/ml of cycloheximide were added to duplicate aliquots of amoebae. The amoebae in suspension remained as single cells throughout incubation. Samples were taken prior to the dispersal of the aggregates and after 4–6 h for determining regulatory and catalytic subunit activities.

**Rate of Synthesis of Regulatory Subunit**—Amoebae were plated at a density of 6 × 10⁶ cells/cm² on Millipore filters supported by pads saturated with PDF buffer (9). They were then incubated with 150 μCi of [3H]leucine/μl either continuously between 6 and 12 h of development (Fig. 5) or for discrete 2-h periods during development (Fig. 6). The labeled amoebae were harvested, washed three times in ME and once in lysis buffer, devoid of the peptide protease inhibitors, and then sonicated, and the debris was removed by sedimentation as described above. The supernatant fraction was treated with preimmune serum and precipitated with heat-killed, formalized, Staphylococcus aureus as described previously (8, 14). The supernatant fluid from that manipulation was then treated with the antisera and centrifuged to remove any regulatory subunit and again precipitated with S. aureus, and the precipitate was washed (8, 14). After boiling in SDS electrophoresis buffer and centrifugation, the samples were subjected to one-dimensional gel electrophoresis (10). The region corresponding to the M₄ = 41,000 regulatory subunit was excised and solubilized for 2 h at 50 °C in 1 ml of NCS tissue solubilizer, and its radioactivity was determined. Counts were corrected for quenching and synthesis of the regulatory subunit was expressed as a percentage of the total trichloroacetic acid-precipitable [3H]leucine incorporated (15) for each sampling point. Parallel gels were subjected to fluorography, using Enlightening according to the manufacturer’s instructions, in order to observe the pattern of the labeled bands. The supernatant obtained after immunoprecipitation was devoid of [3H]leucine-labeled regulatory subunit.

**Materials**—The materials employed were as described in the companion paper (8); in addition, we used peptide protease inhibitors and cycloheximide from Sigma, filters and support pads from Millipore Corp., NCS tissue solubilizer from Amersham Corp., and [3H]leucine and Enlightening from New England Nuclear.

**RESULTS**

**Quantitation of the Regulatory Subunit**—Fig. 1 shows an autoradiograph of photoaffinity-labeled extracts of vegetative (Lane 2) and developing (Lane 3) amoebae. The major band corresponding to M₄ = 41,000 has been identified as the regulatory subunit of the cAMP-dependent protein kinase (8, 16). Photoaffinity-labeling of the protein required light and was competed by cAMP (Lane 4); the protein was recognized by antibody to the D. discoideum regulatory subunit (8). Minor bands corresponding to proteins of M₄ >41,000 and <31,000 may be seen; photoaffinity-labeling of these proteins was not competed by a 200-fold excess of cAMP and the proteins did not react with the specific antisera. These bands were labeled more intensely after removal of endogenous nucleotides by passage of extracts through Sephadex G-25; this removal permitted, presumably, the nonspecific binding of 8N-[^32P]cAMP. Sephadex G-25 chromatography was used to avoid the potential problem of competition by endogenous cAMP with the 8N-[^32P]cAMP for the regulatory subunit. Since bound cAMP dissociates rapidly from the D. discoideum regulatory subunit (17, 18), both free and bound cAMP were assumed to be removed by passage through the matrix. The efficacy of the procedure was demonstrated by finding the same binding activity in duplicate extracts prepared from starved amoebae before and after a 15-min exposure to 1 μM cAMP in vivo.

We reported previously that extracts obtained by one cycle of freezing and thawing exhibited photoaffinity-labeled bands corresponding to molecular weights in the range of 32,000–39,000; these bands were also generated by prolonged storage
of extracts which contained originally the 41,000 regulatory subunit and hence assumed to constitute proteolytic fragments. The fragments of \( M_r = 37,000 \) and 39,000 were labeled specifically by the photoaffinity probe and reacted with the antibody. Similar proteolytic fragments have been observed by other workers (19, 20). Extracts from amoebae starved for longer than 6–8 h either on agar or in suspension and prepared by mild sonication in the presence of 1 mM EDTA, 10 mM benzamidine, and 1 mM PMSF, but in the absence of the protease inhibitor peptides, showed primarily the \( M_r = 41,000 \) band. In the case of vegetative amoebae, however, extraction under these conditions usually yielded only a faint smear in the molecular weight range of 32,000–36,000 (Fig. 1, Lane 1) with much less photoaffinity-labeling than did a parallel sample prepared from amoebae lysed in the presence of the peptide protease inhibitors (Lane 2). It appears that the peptide antipain provided most of the protection. The mixing of extracts from vegetative and starved amoebae prior to lysis indicated that the proteolytic activity of the vegetative amoeba converted all of the \( M_r = 41,000 \) regulatory subunit in the mixture to the faintly labeled 39,000–36,000 smear. Interestingly, this proteolytic activity was largely removed from extracts by centrifugation at 30,000 \( 	imes g \) for 15 min. The regulatory subunit of the cAMP-dependent protein kinase appears to be particularly sensitive to proteolysis; the activity of the catalytic subunit and the pattern of Coomassie-stained proteins in SDS-polyacrylamide gels were unaffected by conditions which led to the destruction of the regulatory subunit. However, the cAMP-independent protein kinase (Pool I, Fig. 2) in extracts of vegetative amoebae was also sensitive to proteolysis. It seems that the relevant proteolytic activity decreases during development and that the appearance of photoaffinity-labeled peptides of a molecular weight of less than 41,000 early in development is an artifact of the extraction procedure reflecting changes in cellular proteolytic activity rather than changes in the sensitivity of the regulatory subunit; it has \( M_r = 41,000 \) throughout development.

The photoaffinity-labeling procedure (0 °C, 90 s total reaction time) was adopted in order to avoid degradation of the \( 8N_3-\left[{ }^{32}P\right]cAMP \) by the \( D. \) discoideum phosphodiesterase which, unlike the mammalian enzyme, hydrolyzes the nucleotide (21), and because essentially maximal labeling of the 41,000 regulatory subunit was achieved under these conditions. Conceivably, another cAMP-binding protein with bound endogenous cAMP, which exchanged only slowly at 0 °C (similar to the behavior of cAMP bound by the mammalian regulatory subunits), might be overlooked by this procedure. Therefore, both the 30,000 \( 	imes g \) supernatant fractions and the pooled proteins, purified through Sephadex G-25, were subjected to the “cold trap” procedure of Hoyer et al. (22); the proteins were incubated with the photoaffinity probe for 30 min at 37 °C and this was followed by a 30-min exposure at 0 °C. No additional specific photoaffinity-labeled bands were uncovered. By the same token, the use of 2 mM \( 8N_3-\left[{ }^{32}P\right] \) cAMP, a concentration often necessary to visualize the mammalian \( R_0 \) subunit, revealed no additional, specifically photoaffinity-labeled bands.

For the quantitative use of the photoaffinity-labeling assay, it was essential that the efficiency of labeling be the same for all samples and that all the labeled material in the \( M_r = 41,000 \) band was indeed the regulatory subunit. The first criterion was met by the demonstration that mixed extracts, obtained from amoebae at different stages of development, always yielded additive amounts of regulatory subunit; furthermore, the ratio of the \( M_r = 41,000 \) regulatory subunit activity, as measured by photoaffinity-labeling to that measured by \( [H]cAMP \) binding was constant, provided that \( [H] \) cAMP binding was measured under conditions which excluded the activity of the low affinity adenine-analog-binding protein (23, 24). Scatchard plots of the amount of photolabeled regulatory subunit (8N3-[\({ }^{32}P\) ]cAMP in the range of 1–1000 nM) yielded the same \( K_d \) of 15–25 nM in samples obtained from vegetative amoebae and from amoebae starved for 6 and 12 h, respectively. The second criterion, i.e. of identity, was met by the demonstration that in samples from all stages only the same two spots were observed in two-dimensional gel electrophoresis, as reported previously (16). Furthermore, antiserum against partially purified regulatory subunit, isolated from developing amoeba (8), precipitated the same \( M_r = 41,000 \) material from vegetative and developing amoeba, irrespective of the time of sampling.

Quantitation of the Catalytic Subunit—The occurrence of significant cAMP-independent protein kinase activity in extracts defeated attempts to quantitate catalytic subunit activity in the 30,000 \( 	imes g \) supernatant fractions. Fig. 2 shows that passage of the supernatant fractions through chromatofocusing columns separated the cAMP-dependent protein kinase from interfering cAMP-independent protein kinases. The run-through fraction (Pool I, Fig. 2) contained protein kinase which catalyzed the phosphorylation of casein more effectively than that of Kemptide. The phosphorylation of Kemptide was not stimulated by cAMP and not affected by the inhibitor of cAMP-dependent protein kinase (Table I); i.e. the activity in this fraction was not that of the catalytic subunit derived from a cAMP-dependent protein kinase. The Kemptide-phosphorylating activity in Pool I was about 90% of that in the cAMP-dependent protein kinase fractions of Pool II which eluted from the column at approximately pH 6.4. The activity of the protein kinase of Pool II was usually stimulated about 5-fold (range of 2–8-fold) by cAMP and the figure.
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Protein kinase assays were performed as described under "Experimental Procedures." A constant amount (500 ng) of purified rabbit inhibitor of the cAMP-dependent protein kinase was used. The cAMP-independent protein kinase was purified from D. discoideum catalytic subunit was purified through the Cm-cellulose column (see Fig. 2). The concentration of the inhibitor of the CAMP-dependent kinase was used. The CAMP-dependent protein kinase was present in extracts of vegetative and starved amoebae, respectively.

Table I

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>Substrate</th>
<th>Activity</th>
<th>Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAMP</td>
<td>pmol/min</td>
<td>%</td>
</tr>
<tr>
<td>Bovine catalytic subunit</td>
<td>Kemptide</td>
<td>17.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Rat cAMP-dependent protein kinase</td>
<td>Histone H1</td>
<td>18.7</td>
<td>2.2</td>
</tr>
<tr>
<td>D. discoideum catalytic subunit</td>
<td>Kemptide</td>
<td>30.5</td>
<td>3.6</td>
</tr>
<tr>
<td>Extract of vegetative amoebae</td>
<td>Casein</td>
<td>31.8</td>
<td>4.4</td>
</tr>
<tr>
<td>Pool 1</td>
<td>Kemptide</td>
<td>8.4</td>
<td>7.7</td>
</tr>
<tr>
<td>Pool 2</td>
<td>Kemptide</td>
<td>6.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Casein</td>
<td>10.0</td>
<td>2.5</td>
<td>25</td>
</tr>
<tr>
<td>Extract of amoebae starved 12 h on agar</td>
<td>Kemptide</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>Pool 1</td>
<td>Kemptide</td>
<td>3.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Pool 2</td>
<td>Kemptide</td>
<td>6.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Casein</td>
<td>18.6</td>
<td>3.2</td>
<td>17</td>
</tr>
<tr>
<td>Extract of amoebae starved 16 h in suspension</td>
<td>Kemptide</td>
<td>4.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Pool 2</td>
<td>Kemptide</td>
<td>15.6</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Kemptide was the preferred substrate; the activity was suppressed by the inhibitor of cAMP-dependent protein kinase (Table I). Photoaffinity labeling with 8Na-[32P]cAMP and two-dimensional electrophoresis of Pool I protein obtained from extracts of vegetative and of starved amoebae demonstrated that Pool I contained proteins corresponding to both spots representing the regulatory subunit. The ratio of protein kinase activities found in the Pool II material in the presence and absence of cAMP cannot be taken as a measure of the in vitro state of dissociation of the enzyme. As noted previously, cAMP is released rapidly from the regulatory subunit in vitro (17, 18); furthermore, the same activity ratio was found for duplicate samples obtained from amoebae sampled prior to, and 15 min after, in vivo exposure to 1 mM cAMP. Nevertheless, the total protein kinase activity found in Pool II, when Kemptide served as substrate and in the presence of cAMP, may be taken as a measure of cAMP-dependent protein kinase catalytic subunit activity in the amoebae. The activity was normalized on the basis of the total amount of sample protein applied to the chromatofocusing column. The recovery of the cAMP-dependent protein kinase activity from the chromatofocusing columns was always at least 70%, and frequently greater than 100%, of the activity in the 30,000 × g supernatant fraction applied to the columns. Conceivably an inhibitory component, such as a phosphoprotein phosphatase, was removed by chromatofocusing.

Development of Amoebae on Agar—The regulatory and catalytic subunit activities found in amoebae developing on agar are shown in Fig. 3. The respective subunit activities of vegetative amoebae were 20-35% of those found in developing cells. The activities of vegetative amoebae were the same, irrespective of the density (in the range of 1-10 × 10^6 amoebae/ml) to which the amoebae were grown. The same levels were observed in AX3 amoebae grown on Escherichia coli for at least 10 generations to a final density of 3 × 10^6 amoebae/ml and in amoebae grown axenically. The approximately parallel increase in the two subunits of the cAMP-dependent protein kinase began early in development (2-4 h) and reached its maximum during aggregation. While the time course of the increase and the final level of subunits varied from experiment to experiment, the same pattern and overall increase was always observed. The decrease at late stages of development (Fig. 3) was variable (see Fig. 5). The concentration of regulatory subunits, estimated from Scatchard plots of [3H]cAMP binding, in vegetative amoebae was approximately 100,000 molecules/amoeba; this corresponds to a concentration of about 0.3 pM. The ratio and the position of the two spots corresponding to the regulatory subunit in two-dimensional electrophoresis were invariant (16).

Development of Amoebae in Suspension—In order to determine whether or not sustained cell-cell contact or the formation of a three-dimensional assembly of amoebae was a prerequisite for the increase in cAMP-dependent protein kinase activity, amoebae were starved, suspended in buffer, and shaken rapidly; pulses of cAMP were administered. Under
These conditions a few of the amoebae formed loose clumps of 2-4 cells; most amoebae remained as single cells. Fig. 4 shows that there was a rapid increase in the activity of the regulatory subunit which eventually reached the same level as that found in amoebae developing on agar. Table II shows the range of the increases in cAMP-dependent protein kinase activity observed in different experiments. Subunit activity was normalized to that found in amoebae developing on agar in the same experiment so as to permit comparison with the results depicted in Figs. 3 and 4. It may be seen, despite the range of values found in different experiments with amoebae starved in suspension, that the increase in the activities of both regulatory and catalytic subunits was at least as large in amoebae starved in the absence of cell-cell contact, even without added cAMP, as it was in amoebae developing on agar; added cAMP led to an even greater increase in the two activities.

Disaggregation of Aggregates—Cyclic AMP has a striking effect on single cell suspensions of amoebae obtained by the dispersion of aggregates at the tip stage of development. When these amoebae are shaken rapidly in the presence of EDTA, many of the mRNAs and proteins formed earlier in the developing aggregates decay over a period of about 5 h. The addition of cAMP at the time of disaggregation, however, prevents the loss of at least certain of the mRNAs and proteins and, furthermore, addition of cAMP after 5 h, i.e. after the loss has already occurred, results in the resynthesis of many of the mRNAs and proteins (3, 4, 7). In view of the possible role of the cAMP-dependent protein kinase as the mediator of these effects of cAMP, it was of obvious interest to study the behavior of the enzyme and its subunits following the dispersal of the aggregates. We found that the subunits of the cAMP-dependent protein kinase remained at the high levels which they had attained during the development of the aggregates even after the disaggregation of the structures. This finding is consistent with the hypothesis that the cAMP-dependent protein kinase mediates the effects of cAMP. Furthermore, the high level of subunit activity was maintained in the presence of cycloheximide, indicating that the cAMP-dependent protein kinase was metabolically stable under the conditions of the experiment.

**Table II**

Activity of the regulatory and catalytic subunits in *D. discoideum* developing in suspension

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Regulatory subunit</th>
<th>Catalytic subunit</th>
</tr>
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<tbody>
<tr>
<td>Vegetative (12-16 h)</td>
<td>24 ± 3 (3)</td>
<td>29 ± 5 (3)</td>
</tr>
<tr>
<td>Agar (12-16 h)</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>Suspension (12-16 h)</td>
<td>97 ± 9 (4)</td>
<td>102 ± 19 (5)</td>
</tr>
<tr>
<td>Suspension + cAMP</td>
<td>132 ± 15 (5)</td>
<td>151 ± 18 (5)</td>
</tr>
<tr>
<td>Suspension + cycloheximide</td>
<td>27</td>
<td>21</td>
</tr>
</tbody>
</table>

**Fig. 5.** Incorporation of [3H]leucine into the regulatory subunit during development of amoebae on Millipore filters. Amoebae developing on four filters were treated with 150 μCi of [3H]leucine/filter from 6-12 h after the start of starvation and harvested and disrupted as described under “Experimental Procedures.” The extract was treated sequentially with preimmune serum (Lane 1) and the antiregulatory subunit serum (Lane 2); the resulting precipitates (obtained with heat-killed, formalized S. aureus) were subjected to gel electrophoresis as described under “Experimental Procedures.” A fluorogram of the resulting gel is shown.

**Fig. 6.** Rates of synthesis of the regulatory subunit of the *D. discoideum* cAMP-dependent protein kinase during development on Millipore filters. For each point, amoebae developing on two filters were exposed to 150 μCi of [3H]leucine/filter for 2 h. At the end of the exposure (shown as the time point in the figure) the amoebae were harvested and the [3H]leucine in the immunoprecipitable regulatory subunit (as percent of [3H]leucine in total protein (O—O)) and photoaffinity-labeled regulatory subunit activity (x—x) were determined as described under “Experimental Procedures.” Maximal regulatory subunit activity was 550 fmol of 8N-[32P]cAMP bound covalently per mg of protein.

determine, whether or not the regulatory subunit of the cAMP-dependent protein kinase was synthesized de novo during amoebal development. The data presented in Fig. 5 show that the regulatory subunit incorporated [3H]leucine during the period of 6-12 h of starvation, i.e. when the rate of increase in regulatory subunit activity, as determined by photoaffinity-labeling with 8N-[32P]cAMP, was maximal. Other labeled protein bands can be discerned (Lane 2); all of these are found also in the *S. aureus* precipitates of reaction mixtures between the labeled extracts and preimmune serum; i.e. the proteins may react with the preimmune serum or they may be adsorbed nonspecifically to the *S. aureus* preparation.

Fig. 6 shows the rate of synthesis of the regulatory subunit, relative to total protein synthesis during development on filters, as determined by [3H]leucine incorporation during 2-h exposures of the amoebae to the labeled amino acid. The maximal rate of synthesis occurs at 8 h of starvation, i.e. at the onset of aggregation. A decrease in the rate of synthesis appears to be followed by a second increase which coincides approximately with culmination (see drawing, Fig. 3). The second increase in the relative rate of synthesis of the regu-
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Discussion

In the preceding paper (8) we characterized the cytosolic cAMP-dependent protein kinase of the cellular slime mold, D. discoideum. In the present communication we describe the behavior of the enzyme during the development of the organism. We find that both regulatory and catalytic subunit activities increase approximately 4-fold during development; our measurements of regulatory subunit activity are more accurate than those of catalytic subunit activity. We conclude, making allowance for the accuracy of the assay procedures, that the two subunits increase in parallel; this suggests an identical mechanism of control for the synthesis of the two proteins. We cannot calculate the number of molecules of catalytic subunits per cell and therefore cannot determine the cellular ratio of the two subunits of the CAMP-dependent protein kinase; their parallel increase indicates that the ratio remains constant during development.

An earlier claim (6), that vegetative amoebae were devoid of cAMP-dependent protein kinase, is incorrect (see Figs. 3, 4, and 6 and Table II); by the same token our earlier estimate (16) of regulatory subunit activity in vegetative amoebae was too low. The discrepancies between the earlier observations and our current findings are explained by the incomplete inhibition of the potentiolytic activity of vegetative amoebae in the earlier experiments. The disruption of the amoebae in the presence of appropriate inhibitors of protein synthesis, particularly antipain, permits the demonstration of significant cAMP-dependent protein kinase activity in vegetative amoebae. Antipain has been reported to protect also the regulatory subunit of the CAMP-dependent protein kinase of the aquatic phycomycete Blastocladiella emersonii against endogenous protease activity (25). The decrease in antipain-sensitive proteolytic activity during development in D. discoideum is similar to the decrease found by Gustafson and Thon (26) for Proteinase I; the relationship, if any, of Proteinase I and of the enzyme responsible for the loss of CAMP-dependent protein kinase activity in vegetative amoebae, remains to be established. There is no evidence for a role of proteolysis in the developmental regulation of CAMP-dependent protein kinase activity. The regulatory subunit of the enzyme shows an invariant Mr. = 41,000, as determined by SDS gel electrophoresis, during the entire course of development, provided that proper precautions are taken during the disruption of the amoebae; lower molecular weight species are proteolytic fragments generated during the disruption of the cells. Likewise the position and the ratio of the two spots on two-dimensional gel electrophoresis, corresponding to the regulatory subunit, remain constant throughout development. The catalytic subunit of the cAMP-dependent protein kinase appears to be more resistant to in vitro proteolysis than the regulatory subunit; we find no evidence for the occurrence of a form of the catalytic subunit with a higher molecular weight than 33,000, as described in the preceding paper (8).

A priori, the increase in the activities of the catalytic and regulatory subunits of the cAMP-dependent protein kinase during development might reflect either the activation or the de novo synthesis of the two proteins. The finding (Fig. 5) that [3H]leucine was incorporated into the regulatory subunit of the enzyme and that cycloheximide blocked the developmental increase of the regulatory (Fig. 4 and Table II) and of the catalytic (Table II) subunit activities indicates that the increase in cAMP-dependent protein kinase activity constitutes de novo synthesis.

It was of obvious interest to determine whether or not sustained cell-cell contact was required for the increase in cAMP-dependent protein kinase activity during development. The data presented in Fig. 4 and Table II demonstrate clearly that cAMP-dependent protein kinase activity increased to the same extent in single cell suspensions of amoebae as in amoebae developing on agar, i.e. under conditions where cell-cell contact was maximal. The exposure of amoebae in suspension to pulses of low concentrations of cAMP caused a further increase in cAMP-dependent protein kinase activity. We cannot state at present, whether this increase in activity is itself mediated by the cAMP-dependent protein kinase or whether the increase is an indirect consequence of the effect of cAMP on overall development.

Amoebae, starved in suspension, i.e. with no sustained cell-cell contact, when treated with cAMP, synthesize a number of mRNAs corresponding to those found later in the anterior portion of the slugs (3, 4). Synthesis of these mRNAs starts at 8–12 h of starvation (3, 4); we find that the synthesis of both subunits of the CAMP-dependent protein kinase starts at 2–4 h of starvation and reaches maximal levels prior to the formation of the tips of the aggregates (Fig. 3), i.e. before the increase in mRNA complexity. This fact makes the newly synthesized CAMP-dependent protein kinase an obvious candidate as the mediator of the effects of cAMP on the synthesis of these mRNA species. It might be mentioned parenthetically that during aggregation cAMP exerts a negative effect on the transcription of the mRNA for discoidin I (27). Another set of developmentally regulated mRNAs is synthesized only after tip formation and is found in the posterior portion of the slugs. Synthesis of this set of mRNAs appears to require cell-cell contact as well as cAMP (3, 4).

The decrease in cAMP-dependent protein kinase activity late in development has been variable (compare Figs. 3 and 6) and the role of cAMP and of the enzyme in late events, particularly culmination, remains to be explored.

It has been shown (3, 4, 7) that certain developmentally regulated mRNAs and proteins disappear when amoebal aggregates at 12–13 h of development are dispersed and incubated as single cell suspensions. Exogenous cAMP prevents that disappearance and brings about the resynthesis of the mRNAs, even if added after these mRNAs have been allowed to disappear (4–5 h in shaking suspensions). The fact that both subunits of the cAMP-dependent protein kinase remained at the levels which they had attained when the aggregates were dispersed (Table II) is compatible with a role of the enzyme in the effect of cAMP in the maintenance and the resynthesis of these developmentally regulated mRNAs. The persistence of the enzyme in the presence of cycloheximide indicates the metabolic stability of the cAMP dependent protein kinase, at least at that particular stage of development.

It is clear that the cAMP-dependent protein kinase of D. discoideum is developmentally regulated; nothing is known about its in vivo state of activation. Although no differences between the cAMP-dependent protein kinase of vegetative and of developing amoebae have been observed, the occurrence of such differences is not excluded. The fact that a
change in cellular cAMP concentration suffices to bring about at least as large an increase in cAMP-dependent protein kinase activity as the 4-fold increase in activity due to the de novo synthesis of cAMP-dependent protein kinase in the present paper, raises questions about the physiological significance of the observed increase. The possibility should be considered, therefore, that the cAMP-dependent protein kinase synthesized during amoebal development is functionally distinct, either qualitatively or with respect to subcellular location, from the enzyme found in vegetative amoebae.

Increases in cAMP-dependent protein kinase activity, quantitatively similar to those observed in the present work, have been noted in differentiating single cells (e.g. 28–30); in no case, however, has a causal link been established between changes in cAMP-dependent protein kinase activity and the events leading to differentiation. The study of *D. discoideum* offers distinct advantages for the exploration of the role of cAMP in morphogenesis and differentiation. It is easy to prepare large quantities of cells, synchronous with respect to their state of development. Mutants, abnormal in development, are readily available. Clones of developmentally relevant mRNAs have been isolated and their occurrence in the anterior (prestalk) versus posterior (prespore) portions of the slug has been established. It is this combination of information on the location of developmentally regulated mRNAs in the slug and on the future fate of the cells carrying the particular mRNAs which makes *D. discoideum* exceptionally suitable for the study of cellular differentiation and its relation to morphogenesis.

Acknowledgments—We thank Dr. B. Haley for gifts of 8N-32P cAMP and instruction in its use, Drs. E. H. Fischer and G. J. Foulkes for the inhibitor of cAMP-dependent protein kinase, Dr. R. L. Erikson for help in the preparation and use of *S. aureus* in immune precipitation, and Drs. T. Chambers and T. Langan for the cAMP-independent rat histone kinase.

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