Biochimica et Biophysica Acta, 567 (1979) 339—346

BBA 68707

PROTEIN KINASES OF *DICTYOSTELIUM DISCOIDEUM*, STRAIN AX-2

HANS JOBST RAHMSDORF * and SHOU-HSANG PAI **

Institut für Biochemie (Naturwissenschaftliche Fakultät) der Universität Innsbruck, Innrain 52, A-6020 Innsbruck (Austria)

(Received September 8th, 1978)

Key words: Protein kinase isoenzyme; Cyclic AMP; (Dictyostelium discoideum)

Summary

In cell homogenates of *Dictyostelium discoideum*, strain AX-2, four major soluble protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) and one membrane-associated protein kinase activity were identified. The enzymes showed high affinity for casein. One of the enzymes was purified by affinity chromatography on casein-coated Sepharose. The soluble high molecular weight enzymes phosphorylated histones, whereas the low molecular weight enzymes did not. The same protein kinase species were present in vegetative and aggregation-competent cells. Their specific activity, however, changed during the development to aggregation competence. None of the enzymes was stimulated by cyclic AMP or cyclic GMP, regardless of their origin from vegetative or aggregation-competent cells.

Introduction

In the cellular slime mould, *Dictyostelium discoideum*, the transition from vegetative growth to aggregation competence is triggered by an extracellular event: the deprivation of food [1]. Cyclic AMP has been proposed as the mediating substance [2]. Indeed, cyclic AMP pulses induced aggregation competence [3,4] and, at the same time, cyclic AMP phosphodiesterase was overproduced [5,6]. Metabolizable sugars in the starvation medium interfered with these processes at a step during cyclic AMP generation [2,7].

* Present address: Kernforschungszentrum Karlsruhe, Institut für Genetik und für Toxikologie von Spaltstoffen, Postfach 3640, D 7500 Karlsruhe 1, F.R.G.

** Present address: Department of Biochemistry, National Defense Medical Center, P.O. Box 7432, Taipei, Taiwan.

Abbreviation: MES, 2-(*N*-morpholino)ethanesulfonic acid.

Since in other eukaryotes, cyclic AMP exerts its action by the well-documented activation of protein kinases (ATP:protein phosphotransferase, EC 2.7.2.37) [8], a role of protein kinases in the induction to aggregation competence seemed plausible. This idea is further supported by the detection of cyclic AMP-dependent protein kinases in the non-axenic *Dictyostelium* strain V12/M2 [9]. Since this would be very important for the interpretation of the role played by cyclic AMP in development, we have examined another strain of *D. discoideum*: the axenic strain AX-2, which can be grown and starved under defined conditions. We have detected protein kinases in cell homogenates and none of these enzymes was stimulated by cyclic nucleotides.

Materials and Methods

Reagents. Cyclic AMP, cyclic GMP, egg white lysozyme, DNAase, RNAase and alkaline phosphatase were from Boehringer (Mannheim). Histone IIa, histone III, histone VII and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma (St. Louis). Casein, ovalbumin and $(NH_4)_2SO_4$ were from Merck (Darmstadt). DEAE-cellulose (DE 52) and the glass fiber filters (GFC) were from Whatman (Clifton, NJ), Sephadex and the CNBr-activated Sepharose 4B from Pharmacia (Uppsala). [γ-^{32}P]ATP was purchased from The Radiochemical Centre (Amersham).

Buffers and Media. Medium 1 : 5 g Difco Yeast extract, 10 g Difco proteose Peptone No. 3, 1.19 g MES, 18 g glucose, 0.1 g penicillin, 0.1 g streptomycine sulfate, 1 l H_2O (pH 6.6); buffer 1: 16.6 mM MES, $2 \cdot 10^{-4}$ M $CaCl_2$ (pH 6.2); buffer 2: 10 mM $MgCl_2$, 10 mM Tris-HCl , 22 mM NH_4Cl, 1 mM dithiothreitol, 5% glycerol (pH 7.5); scintillation fluid: 1 l toluene, 3.6 g PPO, 0.9 g POPOP.

Culture and Development. The strain used was *D. discoideum* AX-2 [10]. The cells were grown at 23°C on a rotary shaker in medium 1 and harvested at $4 \cdot 10^6$—$6 \cdot 10^6$ cells/ml. Development to aggregation competence was induced according to Malchow et al. [11] in buffer 1, and tested by plating the cells on Millipore filters.

Cell homogenization and protein kinase assay. Vegetative or developing cells were harvested by centrifugation, washed once with buffer 2 and sonicated on ice for 10 s with a Branson B12 sonifier with microtip, at maximum output. The protein kinase assays were done in a total volume of 100 μl containing 20—50 μl enzyme, 0.5 mg/ml casein, 10 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5, 11 mM NH_4Cl, 2.5% glycerol, 0.5 mM dithiothreitol, 0.1 mM EDTA, $1 \cdot 10^{-5}$ M cyclic AMP and 47 μM ATP (106 Ci/mol [γ-^{32}P]ATP). The reaction was started by the addition of enzyme, incubated for 10 min at 25°C and stopped with 1 ml 10% trichloroacetic acid. The samples were boiled for 10 min in trichloroacetic acid to destroy nucleic acids, collected on glass fiber filters and washed 3 times with 10 ml 10% trichloroacetic acid. The filters were washed with CH_3OH and $CH_3OH/CHCl_3$ (1 : 2, v/v). After drying, the radioactivity of the filters was determined and the values given are calculated for 10 min incubation.

Results and Discussion

Separation and purification of the protein kinases

Cell homogenates of *D. discoideum* contained protein kinase activity with high affinity for casein. If the total homogenate was separated by centrifugation for 15 min at 10 000 X *g*, into a soluble and a particulate fraction, and the particulate fraction reextracted by washing and sonication, about 80% of the total protein kinase activity appeared in the soluble fraction. Treatment of the cellular debris with 2% Triton X-100, for 2 h in the cold, did not release further enzyme activity, but yielded a membrane preparation with high-specific protein kinase activity. The soluble protein kinase activity was pooled and subfractionated:

DEAE-cellulose chromatography resolved the preparation into two activities, A and B. Protein kinase activity A did not bind to DEAE-cellulose, whereas activity B eluted at salt concentrations between 0.05 and 0.25 M. (Fig. 1).

Sephadex G-200 chromatography separated enzyme A from the bulk of the other proteins and further resolved the activity into A_1 and A_2. (Fig. 2). For A_1 and A_2, molecular weights of 120 000 and 10 500 were calculated respectively. Activity B was eluted from Sephadex G-200 with the void volume if the chromatography was performed with buffer 2. Upon elution with 0.5 M NaCl, two activities (B_1 and B_2) of 180 000—260 000 daltons and 50 000—80 000 daltons were resolved from the main protein peak (Fig. 3).

Casein-Sepharose chromatography. The protein kinase of *D. discoideum* showed high affinity for casein. One of the enzyme activities, protein kinase

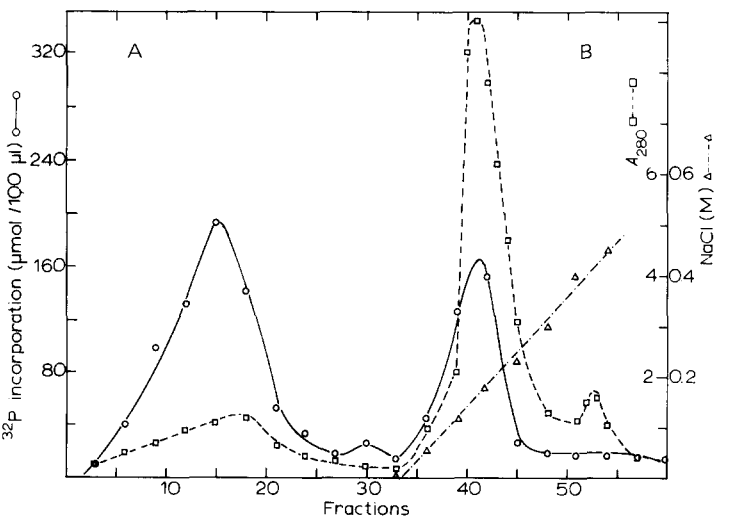

Fig. 1. DEAE-cellulose chromatography of the soluble protein kinase of *D. discoideum* AX-2. The soluble protein kinases from $4 \cdot 10^9$ vegetative cells were diluted with buffer 2 to 100 ml and pumped on a 25 g DEAE-cellulose column equilibrated with buffer 2. The column was washed with buffer 2 until no more protein could be eluted. The column was developed with a linear 400 ml gradient of 0—0.6 M NaCl in buffer 2 (flow rate, 60 ml/h; 8-ml fractions). 50-µl aliquots of each fraction were assayed for protein kinase activity in the presence of cyclic AMP with casein as a substrate. The protein content was monitored by its absorption at 280 nm, and the elution gradient was determined with a conductimeter.

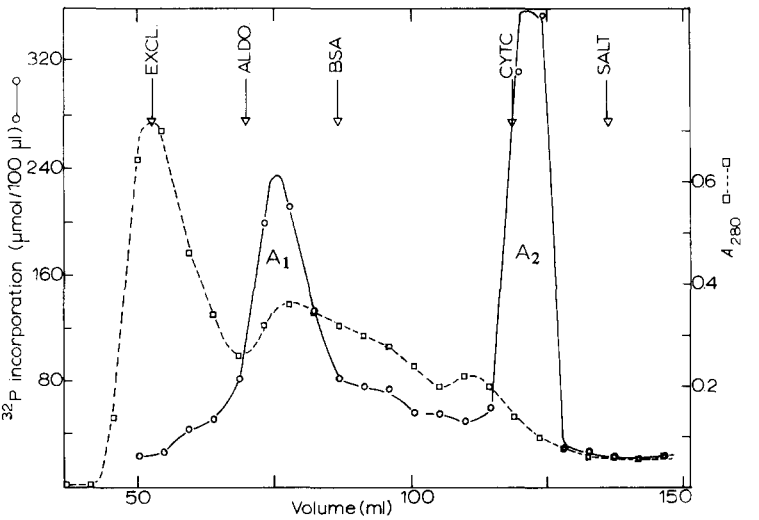

Fig. 2. Sephadex chromatography of protein kinase activity A from *D. discoideum* AX-2. The protein kinase-containing fractions not binding to DEAE-cellulose (fraction A) were concentrated by precipitation with 70% $(NH_4)_2SO_4$, resuspended in 2 ml buffer 2 and layered on a Sephadex G-200 column (100 × 1 cm) equilibrated with buffer 2. The column was eluted with buffer 2 (flow rate, 4 ml/h; 4.5-ml fractions). The protein kinase assay was done in the presence of cyclic AMP with 50 µl enzyme and casein as substrate. The elution profile of the proteins was determined by their absorption at 280 nm. Exclusion volume and salt front were determined by chromatography of a mixture of Dextran blue and *p*-nitrophenol. The column was standardized with catalase, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*.

A_2, was further purified by chromatography on casein-coated Sepharose. Enzyme A_2 bound quantitatively to the column material and was eluted as a single peak of activity at 0.45 M NaCl (Fig. 4). This resulted in an overall purification of enzyme A_2 of about 50-fold, not taking into account the loss of enzyme activity during the purification procedure.

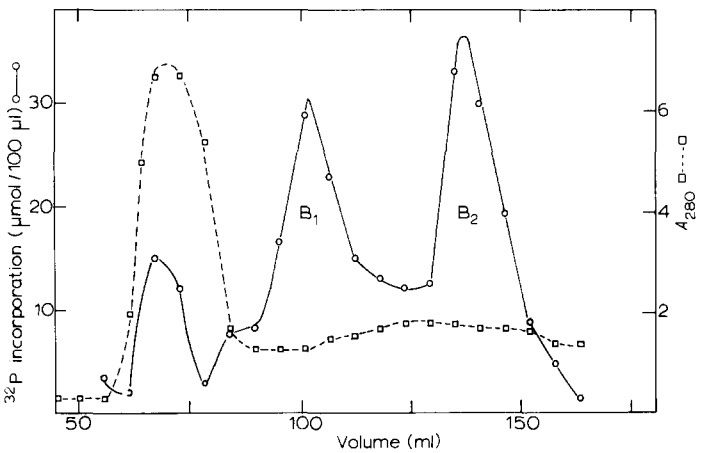

Fig. 3. Sephadex chromatography of protein kinase activity B from *D. discoideum* AX-2. The protein kinase fractions eluted with salt from the DEAE-cellulose column were concentrated by precipitation and chromatographed on a Sephadex G-200 column (120 × 1.2 cm) equilibrated with buffer 2, 0.5 M NaCl. Enzyme tests were done with 20 µl enzyme as described in the legend to Fig. 2.

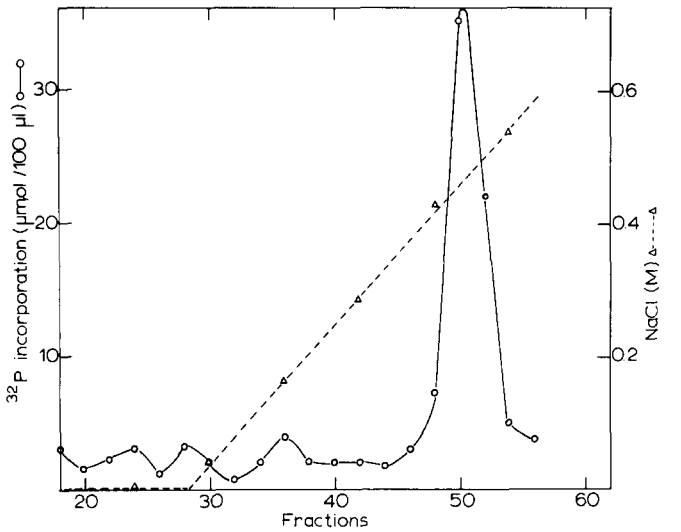

Fig. 4. Affinity chromatography of protein kinase activity A_2 from *D. discoideum* on casein-Sepharose. The casein-Sepharose column was prepared as follows: 5 g CNBr-activated Sepharose 4 B were swollen and washed in 2 l 1 mM HCl. 100 mg casein were dissolved in 50 ml 0.1 M $NaHCO_3$, 0.5 M NaCl. Because casein did not dissolve in this buffer, the pH was raised to 13 and then readjusted with HCl. The casein solution and the Sepharose were mixed and stirred for 16 h in the cold. The suspension was washed with 0.1 M $NaHCO_3$, 0.5 M NaCl and stirred afterwards in 1 M ethanolamine (pH 8.0) for 2 h, followed by two washes with 0.1 M acetate buffer (pH 4.0), 1 M NaCl and 0.1 M borate buffer (pH 8.0), 1 M NaCl. Before packing, the material was equilibrated with buffer 2. The protein kinase A_2-containing fractions were pooled (Fig. 2) and pumped onto the column (0.7 × 5 cm). The column was washed with 40 ml buffer 2 and the enzyme eluted with a 60 ml gradient (0—0.6 M NaCl in buffer 2). Enzyme tests were done with 50 µl enzyme as described in the legend to Fig. 2.

Substrate specificity

The protein kinases of *D. discoideum* phosphorylated a variety of cellular proteins, as shown by electrophoresis and autoradiography of the phosphorylated products on polyacrylamide gels [12]. In addition, all protein kinases phosphorylated casein with high affinity. Histone IIa was phosphorylated only by the high molecular weight protein kinases A_1 and B_1, not by the enzymes A_2 and B_2. None of the enzymes phosphorylated histone III, protamine sulfate, lysozyme or ovalbumin (Table I). The membrane protein kinase phosphorylated casein, histone II A, and very effectively, endogenous membrane proteins.

The phosphorylated material was resistant to DNAase and RNAase but was degraded by pronase. As with most other protein kinases, the amino acids, serine or threonine, accepted the phosphate group. This was shown by the stability of the bond against acid and its instability towards alkaline phosphatase and alkali.

Changes of protein kinase activity during development

The protein kinases isolated from vegetative cells did not differ qualitatively from the protein kinases from aggregation-competent cells. All detectable soluble protein kinases and the intracellular membranal protein kinase were present in both stages of development (see also ref. 17) and behaved identically with respect to substrate specificity.

TABLE I

SUBSTRATE SPECIFICITY OF THE PROTEIN KINASES OF *D. DISCOIDEUM* AX-2

50 μl A_1 or A_2 and 20 μl B_1 or B_2, respectively, of the enzymes pooled after Sephadex G-200 chromatography were assayed with various substrates in the standard enzyme assay. The enzymes were prepared from vegetative cells.

Substrate	Final concentration (mg/ml)	pmol ^{32}P incorporated/100 μl enzyme			
		A_1	A_2	B_1	B_2
—	—	9.0	1.5	6.8	3.9
Casein	0.5	45.7	26.0	38.1	69.6
Histone IIa	2.0	20.5	1.7	13.6	3.5
Histone III	2.0	7.6	2.2	6.7	3.4
Protamine sulfate	2.0	7.5	1.7	4.2	2.0
Lysozyme	2.0	8.3	2.0	10.1	2.2
Ovalbumin	2.0	6.7	2.3	7.0	3.7

In quantitative terms, the total intracellular protein kinase activity did not raise more than by a factor of 1.5 during the acquisition of aggregation competence. The specific activity of A_1 decreased by a factor of two during the development to aggregation competence, measured either with casein or with histone as substrates (Table II). This was compensated, in some experiments, by an increase of the specific activity of A_2.

Lack of response to cyclic nucleotides

None of the protein kinases described here depended on cyclic AMP or cyclic GMP. This is shown (Table III) for the purified soluble protein kinases A_1, A_2, B_1 and B_2 from vegetative cells. To exclude the possibility that during the purification procedure the catalytic and the regulatory subunit of the protein kinases were separated and therefore the dependence on cyclic nucleotides lost, we tried to stimulate the enzymes in whole cell homogenates and in membrane preparations: No stimulation by cyclic nucleotides was found. Also, the phosphorylation of endogenous proteins was not stimulated nor did new bands occur (as examined by slab gel electrophoresis and autoradiography). Thus,

TABLE II

CHANGE IN THE SPECIFIC ACTIVITY OF PROTEIN KINASE A_1 DURING THE DEVELOPMENT OF AGGREGATION COMPETENCE OF *D. DISCOIDEUM* AX-2

Protein kinase A_1 was prepared by DEAE-cellulose and Sephadex G-200 chromatography from vegetative cells and from cells starved for 9 h. The kinase A_1 containing fractions were pooled and the enzyme activity determined with 0.5 mg/ml casein and with 2 mg/ml histone IIa as substrates.

	Substrate	Specific activity (pmol ^{32}P incorporated/mg protein)	
		Vegetative cell	Aggregation-competent cells
Expt. 1	Casein	457	208
	Histone IIa	205	133
Expt. 2	Casein	1032	372
	Histone IIa	440	232

TABLE III

LACK OF DEPENDENCE ON CYCLIC NUCLEOTIDES OF THE PROTEIN KINASES OF *D. DISCOIDEUM* AX-2

Enzymes were the same as in Table I. Casein was used as substrate. Similar results were found with enzymes from developing cells.

Addition	Final concentration (M)	pmol ^{32}P incorporated/100 μl enzyme			
		A_1	A_2	B_1	B_2
—	—	45.7	26.0	38.1	69.6
Cyclic AMP	$1 \cdot 10^{-6}$	48.5	30.0	38.7	81.1
Cyclic AMP	$1 \cdot 10^{-4}$	32.0	32.9	35.7	76.3
Cyclic GMP	$1 \cdot 10^{-6}$	44.7	33.4	30.0	82.5
Cyclic GMP	$1 \cdot 10^{-4}$	51.6	33.5	36.7	90.2

unless the protein kinase has very peculiar properties as compared with mammalian enzymes no cyclic AMP-dependent enzyme existed in vegetative cells of strain AX-2.

To check whether new cyclic AMP-dependent protein kinases are induced during the development of this strain, the soluble protein kinases were isolated from cells at different stages during the acquisition of aggregation competence (2, 4 and 7 h after the start of starvation), separated by DEAE-cellulose chromatography, chromatography on C^6-amino alkyl agarose [13] or isoelectric focussing and tested fraction by fraction for the stimulation by cyclic AMP or cyclic GMP. Substrates were casein and, in addition, histone IIa and histone VII. In no case could stimulation of enzyme activity by cyclic AMP or cyclic GMP be found. An authentic highly purified cyclic AMP-dependent protein kinase from calf ovaries added to the tests was full stimulated by cyclic AMP. The protein kinase inhibitor which interferes exclusively with the catalytic subunit of cyclic AMP-dependent protein kinase (and not with a cyclic AMP-independent enzyme [14]) did not depress enzyme activity. To exclude that high cellular levels of cyclic AMP concealed the requirement for cyclic nucleotides, we lowered the endogenous cyclic AMP levels by a variety of treatments: in vivo by gassing the cells for 10 min with N_2 before homogenization and extraction of the enzymes, in vitro by dialyzing the enzyme preparations extensively or passing them through Sephadex G-25. None of these treatments revealed a dependence of the enzyme on cyclic AMP or cyclic GMP. Cellular cyclic AMP phosphodiesterase or other possible endogenous inhibiting activities did not interfere with the test: (i) the cyclic AMP-dependent protein kinase from calf ovaries was stimulated by cyclic AMP even when added to the incubation mixtures; (ii) the phosphodiesterase was separated by the chromatographic procedures from the protein kinases; (iii) added cyclic AMP at high concentrations was inhibitory rather than stimulatory to the protein kinases. Because at least the membrane cyclic AMP phosphodiesterase is inhibited by high concentrations of cyclic GMP [15] or dithiothreitol [16], we tested the effects of these treatments and found that there was no effect with respect to cyclic AMP requirement of protein kinases.

These results are in contrast to the findings with *D. discoideum* strain V12/

M2, which does not grow axenically. In V12/M2, two cyclic AMP-dependent protein kinases occur early in development [9]. They represent about 10—20% of the total protein kinase activity present in these cells 1—2 h after the beginning of starvation, and their amount decreases during the further development of the cells. The reasons for the differences are unknown. Since we tested under conditions [9] identical to those required for cyclic AMP-dependent protein kinase in V12/M2, we favor the interpretation that, in AX-2, no such enzyme exists and that regulation occurred by some other mechanism. We found recently that *D. discoideum* cells contain three soluble cyclic AMP-binding proteins and one cyclic GMP-binding protein [17]. These proteins do not chromatograph with the soluble protein kinases. It is possible that in *Dictyostelium*, as in *Escherichia coli*, cyclic nucleotides interfer with cellular development by binding to these proteins.

Acknowledgements

Part of the work was supported by a fellowship of the 'Deutsche Forschungsgemeinschaft' to H.J.R. We thank Drs. Rickenberg, Schweiger, Ponta, Gerisch and Malchow for many helpful discussions. We are indebted to Dr. Salokangas from the 'Hormonlabor des Kantonspitals Basel' for the preparation of the cyclic AMP-dependent protein kinase from ovaries and for the electrophoretic analysis of the *D. discoideum* protein kinases and to Ms. E. Bechtel for the preparation of the protein kinase inhibitor.

References

1 Loomis, W.F. (1975) Dictyostelium discoideum, A Developmental System, Academic Press, New York
2 Rahmsdorf, H.J., Cailla, H.L., Spitz, E., Moran, M.J. and Rickenberg, H.V. (1976) Proc. Natl. Acad. Sci. U.S. 73, 3183—3187
3 Darmon, M., Brachet, P. and Pereira da Silva, L.H. (1975) Proc. Natl. Acad. Sci. U.S. 72, 3163—3166
4 Gerisch, G., Fromm, H., Huesgen, A. and Wick, U. (1975) Nature 255, 547—549
5 Klein, C. (1975) J. Biol. Chem. 250, 7134—7138
6 Klein, C. and Darmon, M. (1977) Nature 268, 76—78
7 Rickenberg, H.V., Rahmsdorf, H.J., Campbell, A., North, M., Kwasniak, J. and Ashworth, J.M. (1975) J. Bacteriol. 124, 212—219
8 Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763—3774
9 Sampson, J. (1977) Cell 11, 173—180
10 Watts, D.J. and Ashworth, J.M. (1970) Biochem. J. 119, 171—174
11 Malchow, D., Nägele, B., Schwarz, H. and Gerisch, G. (1972) Eur. J. Biochem. 28, 136—142
12 Rahmsdorf, H.J., Malchow, D. and Gerisch, G. (1978) FEBS Lett. 88, 322—326
13 Rangel-Aldao, R. and Rosen, O.M. (1976) J. Biol. Chem. 251, 3375—3380
14 Walsh, D.A., Ashby, C.D., Gonzales, C., Calcins, D., Fischer, E.H. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1977—1985
15 Malchow, D. and Gerisch, G. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2423—2427
16 Henderson, E.J. (1975) J. Biol. Chem. 250, 4730—4736
17 Rahmsdorf, H.J. and Gerisch, G. (1978) Cell Differ. 7, 249—257