Increased phosphorylation of a ribosomal protein during aggregation of the slime mold *Dictyostelium discoideum*

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A single ribosomal protein (M_r 32 000) becomes phosphorylated during differentiation of *Dictyostelium discoideum* amoebae. This protein is tentatively identified as the 40 S ribosomal protein S_6. Phosphorylation of S_6 is monitored by incorporation of $^{32}P_i$ and by two-dimensional polyacrylamide gel electrophoresis. S_6 is minimally phosphorylated in growing cells. Upon starvation, S_6 is progressively phosphorylated, the degree of phosphorylation being maximal during the aggregation phase of the developmental cycle.

Ribosomal protein *Protein phosphorylation* *Phosphoprotein* Dictyostelium discoideum

1. INTRODUCTION

Protein S_6 of the small (40 S) ribosomal subunit is the major phosphoprotein of mammalian ribosomes [1] and can be phosphorylated at multiple sites both in vitro and in vivo [1,2]. In lower eukaryotic cells, namely *Tetrahymena pyriformis* [3], *Saccharomyces cerevisiae* [4], *Physarum polycephalum* [5] and *Mucor racemosus* [6], a basic protein of the 40 S ribosomal subunit has also been found to be phosphorylated under certain conditions. Comparison of the aminoacid sequence predicted from the nucleotide sequence [7] with the primary structure data for rat liver ribosomal protein S_6 [8], suggests that the 40 S ribosomal phosphoprotein of *Saccharomyces* is probably analogous to mammalian S_6 [7].

In cultivated mammalian cells, increased phosphorylation of S_6 occurs during stimulation of cellular proliferation [9]. All data presently available are consistent with the proposal that S_6 phosphorylation promotes an enhanced rate of protein synthesis [2]. A current hypothesis is that phosphorylated 40 S subunits form initiation complexes more efficiently than non-phosphorylated 40 S subunits [10]. An apparent exception to the rule that S_6 phosphorylation correlates with the growth state has been encountered in the case of *Tetrahymena*, where phosphorylation of a small ribosomal subunit protein is stimulated by starvation [3,11]. In view of these apparently anomalous results with *Tetrahymena*, we decided to study phosphorylation of ribosomal proteins in another lower eukaryote, the cellular slime mold *Dictyostelium discoideum*, where starvation triggers the differentiation of amoebae into aggregation competent cells [12].

The slime mold *D. discoideum* contains ribosomal proteins similar to those of other higher eukaryotes [13] and is an attractive system for studying the phosphorylation of ribosomal protein S_6 during development. In a study of ribosomal proteins during differentiation of *D. discoideum*, authors in [14] found significant changes in several proteins that were associated with specific stages of cell development. Apparently, these changes were

Abbreviations: Mes, 4-morpholineethanesulfonic acid; SDS, sodium dodecyl sulfate

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not due to chemical modifications of pre-existing proteins [15].

Here we show that a single 40 S ribosomal protein, tentatively identified as S_6, was progressively phosphorylated during differentiation of *D. discoideum* amoebae. Phosphorylation of S_6 was monitored by incorporation of $^{32}P_i$ and by two-dimensional polyacrylamide gel electrophoresis. S_6 was minimally phosphorylated in growing cells. Upon starvation, S_6 was progressively phosphorylated, the degree of phosphorylation being maximal during the aggregation phase of the developmental cycle.

2. MATERIALS AND METHODS

2.1. *Cell culture and starvation conditions*

Ax-2 amoebae were maintained as exponentially growing cultures ($1–2 \times 10^6$ cells/ml) in HL-5 medium [16]. Amoebae were starved by washing twice with 17 mM potassium phosphate buffer (pH 6.4) or 20 mM Mes buffer (pH 6.4), resuspending the cells in the same buffer at a density of 10^7 cells/ml, and maintaining them in suspension by agitation [17]. Labeling with $^{32}P_i$ was carried out in Mes buffer by adding carrier-free [^{32}P]orthophosphate at a final concentration of 100–200 μCi/ml. The differentiation of amoebae to aggregation competence was monitored by plating an aliquot of cells onto tissue culture dishes and observing their morphology [18]. Cells which were elongated and immediately formed polar cell contacts were considered aggregation-competent.

2.2. *Preparation of ribosomes and ribosomal proteins*

In a typical preparation, 6×10^7 cells were frozen at different times, thawed in 1 ml of 20 mM Tris–HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 50 mM NaF, 6 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride, 5% sucrose and 0.5% (w/v) Nonidet P40. The lysate obtained by stirring in a vortex mixer for 2–3 min was centrifuged at 20 000 × g for 10 min and the supernatant layered on an equal volume of the extraction buffer containing 10% sucrose. After centrifugation for 3 h at 38 000 rev./min in a Spinco type 50 rotor, the pellet was rinsed and resuspended in 0.1 ml of 0.2 M $MgCl_2$. Ribosomal proteins were immediately extracted with glacial acetic acid and precipitated with acetone as in [13].

Proteins were extracted from whole cells by thawing frozen amoebae in 0.2 ml of 0.2 M $MgCl_2$, followed immediately by addition of 2 volumes of glacial acetic acid. The cell lysate was stirred for 45 min in the cold; RNA was removed by centrifugation at 20 000 × g for 20 min. Proteins were precipitated from the supernatant by addition of 5 volumes of acetone.

Ribosomal subunits were prepared as in [13] and precipitated with ethanol.

2.3. *Two-dimensional polyacrylamide gel electrophoresis*

Ribosomal proteins were identified according to the procedure in [19], as modified by the authors in [20], except that the proteins were dissolved in 10 M urea. Electrophoresis in the first-dimension was performed on 5% acrylamide gels (0.3 × 12 cm) at 90 V for 20 h. The gels were equilibrated in soaking solution containing 10% glycerol for 20 min and the second-dimension run on a 18% acrylamide gel (14 × 15 × 0.1 cm) at 140 V for 18 h.

Ribosomal proteins were also run on the two-dimensional gel system in [13], which combines a separation at pH 4.5 in the first-dimension with a size-based separation in a SDS-gel in the second-dimension.

Gels were stained in 0.1% Coomassie brilliant blue R-250 (Sigma) in 50% methanol, 5% acetic acid and destained in 7% methanol/7% acetic acid. Gels containing samples labeled with ^{32}P were autoradiographed with Kodak X-Omat film and a Cronex Quanta II intensifying screen (Du Pont).

2.4. *Phosphoamino acid determination*

After separation of the ribosomal proteins by two-dimensional electrophoresis, S_6 was eluted from the gel and treated with trypsin (DPCC-treated, from Sigma) as in [21]. The peptides were hydrolyzed with 6 N HCl (2 h, 110°C) and the hydrolysates analyzed by high voltage electrophoresis as in [22]. The markers phosphoserine and phosphotreonine were obtained from Sigma and phosphotyrosine was synthesized as in [23].

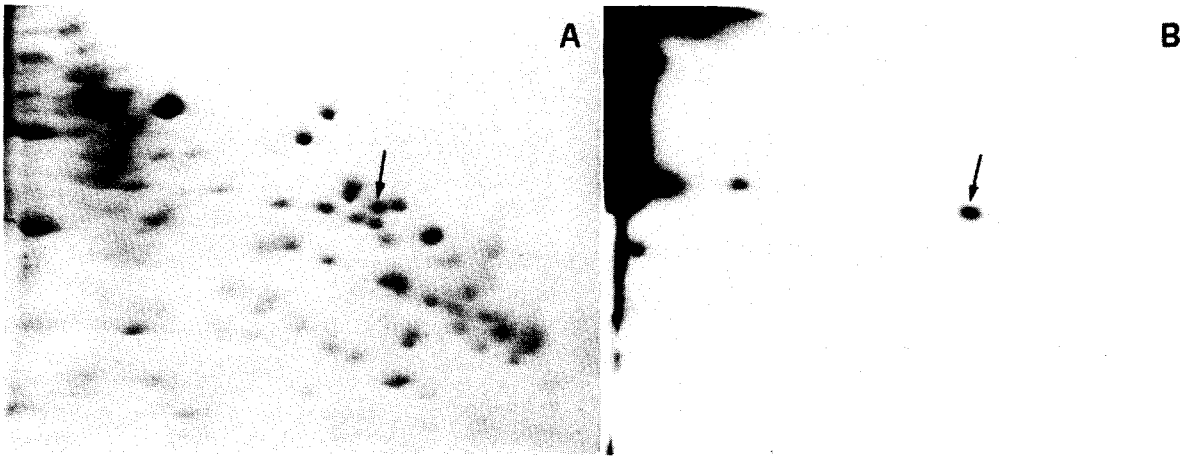

Fig. 1. Coomassie-stained two-dimensional gel (A) and corresponding autoradiogram (B) of ^{32}P-labeled whole-cell proteins from amoebae starved for 3 h. Whole-cell proteins were separated by two-dimensional polyacrylamide gel electrophoresis as in [13]. The arrows indicate proteins S_6 (S_{19} according to the nomenclature in [13]).

3. RESULTS AND DISCUSSION

Phosphorylation of ribosomal proteins from *D. discoideum* was studied by starving amoebae in the presence of $^{32}P_i$, followed by extraction of whole-cell proteins with glacial acetic acid, precipitation of the soluble proteins with acetone and analysis by electrophoesis in two-dimensional gels (acid/SDS) [13]. This approach has the advantage of avoiding dephosphorylation or proteolytic degradation which might occur upon more extensive purification of ribosomes, while still permitting clean separation of the majority of the ribosomal proteins from non-ribosomal proteins by the system of two-dimensional polyacrylamide gel electrophoresis [13,24]. Fig. 1 compares the Coomassie-stain pattern with the corresponding autoradiogram of the whole-cell proteins from amoebae starved for 3 h. Some labeling is observed on the acidic part of the gel, which corresponds to non-ribosomal proteins. In addition, there is a single labeled spot in the center of the autoradiogram, corresponding to the ribosomal protein S_{19} in the nomenclature in [13]. The M_r of this protein is about 32 000. Phosphorylation of this ribosomal protein could be observed even after only 30 min of starvation and increased during the starvation period as shown in table 1.

That the increase in labeling of this ribosomal protein indeed reflects a net change in the phosphorylation state as opposed to a mere increase in the specific activity of the precursor pool, was demonstrated by analyzing the ribosomal proteins in the electrophoresis system in [19] as modified by the authors in [20]. This widely used two-dimensional gel electrophoresis system (basic in the first-dimension and acid in the second-dimension) permits not only correlation of the ribosomal proteins from *D. discoideum* with the uniform nomenclature proposed for mammalian ribosomal proteins [25], but also separation of the

Table 1

Phosphorylation of S_6 during differentiation

H of starvation	Cpm incorporated into S_6 (S_{19})[a]
0.5	430
1	510
2	715
4	925
6	1080
8	1120

[a] Ribosomal proteins were separated by two-dimensional polyacrylamide gel electrophoresis [13]. The region containing S_6 was excised and the radioactivity determined. The blank correction was obtained by counting an equivalent section of gel. The blank values were 80–100 cpm

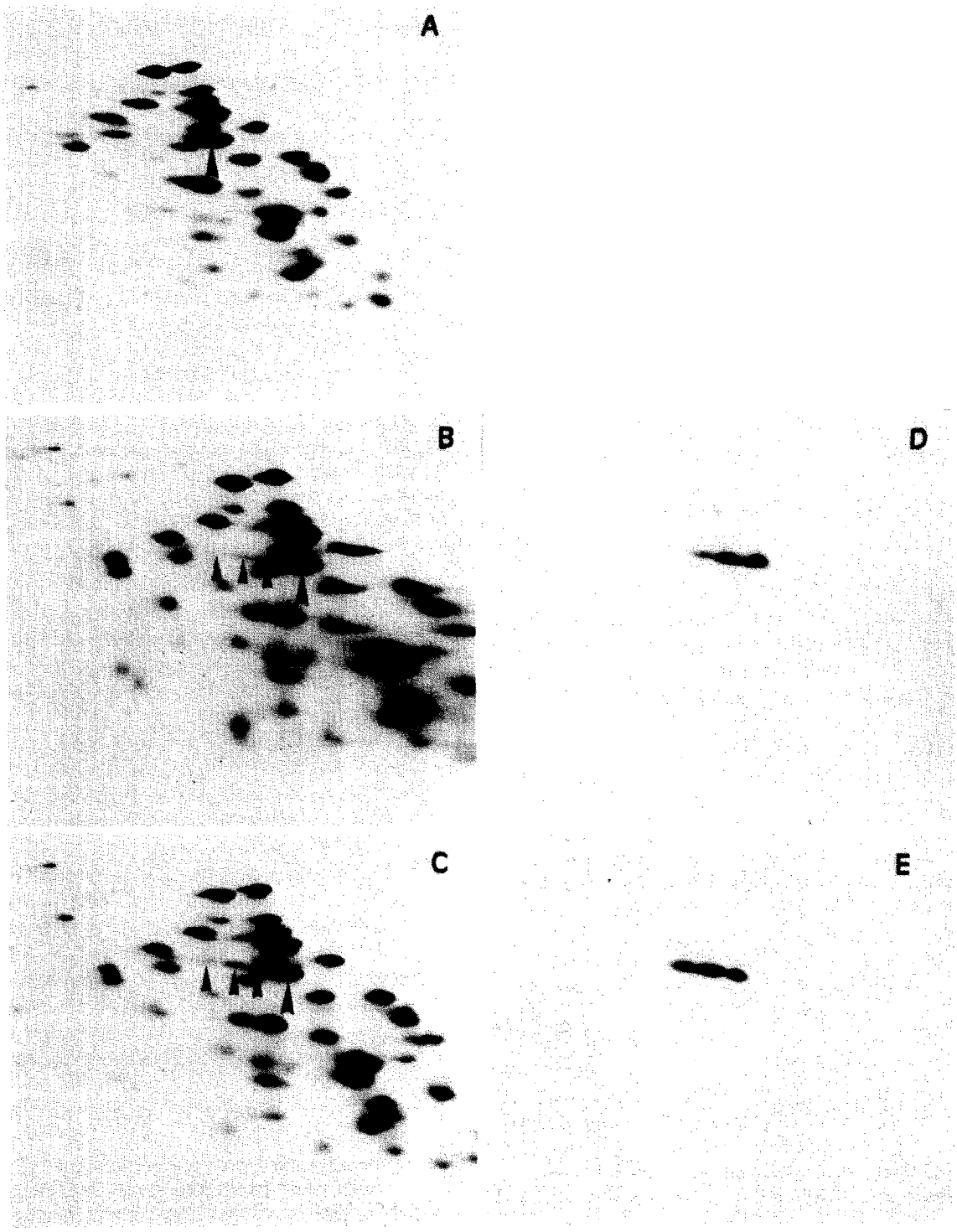

Fig. 2. Coomassie-stained two-dimensional gels of ribosomal protein isolated from growing cells (A) and amoebae starved for 2 h (B) and 6 h (C) according to the system in [20]. (D) and (E) are autoradiograms of the electrophoretograms shown in (B) and (C), respectively. The larger arrowhead indicates non-phosphorylated S_6 and the smaller arrowheads indicate phosphorylated derivatives of S_6.

phosphorylated derivatives of S_6 [9,26]. The problem of dephosphorylation was circumvented by rapidly isolating the ribosomes in the presence of NaF as described in section 2.

Fig. 2 presents ribosomal protein patterns obtained by the technique in [19] from growing cells and from amoebae starved for 2 or 6 h. Comparison of the Coomassie blue and autoradiography patterns indicates that a single ribosomal protein is phosphorylated during starvation. This protein is identified as S_6. This tentative identification with the corresponding protein of animal ribosomes [25] is based on its M_r-value, its relative position in the two-dimensional electropherogram of ribosomal proteins and its changing degree of phosphorylation. The protein S_6 was also unambiguously identified from a 40 S subunit pattern (not shown).

As shown in [9,26], an increasing degree of phosphorylation of S_6 results in slower migration in both dimensions of the electrophoresis. In this regard, fig. 2 indicates an anodal shift of S_6 with progressive cell differentiation. This anodal shift of S_6 on autoradiograms points to a quantitative increase in the amount of phosphate incorporated into S_6. An anodal shift of S_6 could also be detected on Coomassie-stained patterns (fig. 2B and C), implying a redistribution in the relative proportions of the S_6 derivatives accompanying the extent of phosphorylation. In growing cells, most of S_6 migrated at the unphosphorylated native position (fig. 2A). Two h after starvation however, a part of S_6 had shifted to more anodic positions (fig. 2B). At later times, the shift in the migration of S_6 toward the more highly phosphorylated derivatives was even more pronounced (fig. 2C). At least 3 discrete derivatives of S_6 could be resolved (fig. 3) in ribosomes of aggregation-competent amoebae. Treatment of ribosomes from starved cells with alkaline phosphatase resulted in a return of the electrophoretic mobility of this protein to that found in growing cell ribosomes (not shown).

Mixing (1 : 1 ratio) of growing cells with starved cells for 8 h prior to the preparation of ribosomes did not produce any change in the extent of S_6 phosphorylation (beyond that expected on the basic of simple dilution). Analysis of hydrolysates of S_6 by high voltage electrophoresis [22] revealed that most, if not all, of the phosphate associated with S_6 is present as *o*-phosphoserine (fig. 4).

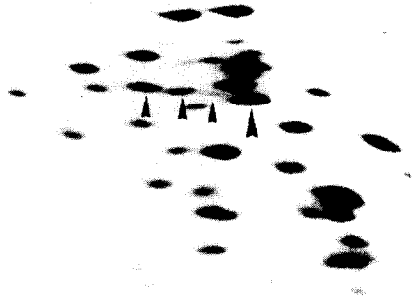

Fig. 3. Coomassie-stained two-dimensional gel of ribosomal protein isolated from competent aggregation amoebae. Cells were starved in 17 mM phosphate buffer (pH 6.4) for 8 h, ribosomes isolated, and ribosomal proteins analyzed by the technique in [20]. Arrowheads as in fig. 2.

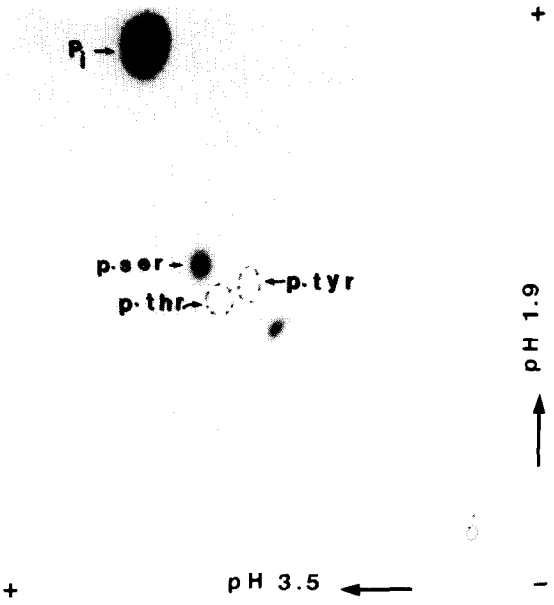

Fig. 4. Phosphoamino acids from ribosomal protein S_6.S_6 was isolated from cells starved for 7 h as described in section 2. The phosphoamino acids were separated on cellulose thin-layer plates by electrophoresis at pH 1.9 for 4 h at 0.5 kV and at pH 3.5 for 90 min at 0.5 kV. The positions of ninhydrin-stained phosphoamino acids are indicated.

These results demonstrate that S_6, though minimally phosphorylated in growing cells, is extensively phosphorylated when the amoebae differentiate. Phosphorylation of S_6 was maximal at the time when cells expressed aggregation competence (6 h of starvation). This phosphorylation level persisted for about 2–4 h, during which time cells entered into compact aggregates, followed by a progressive decline. At the culmination stage, the phosphorylation of S_6 was again minimal. When cell differentiation was inhibited by the presence of a complete aminoacid mixture [27,28] no net phosphorylation of S_6 was observed. Moreover, starvation per se is not responsible for the observed phosphorylation since starvation of the aggregateless mutant Agip 53 [29] did not induce phosphorylation of S_6 (unpublished).

The failure of authors in [14,15] to detect S_6 phosphorylation during differentiation is probably a consequence of the two-dimensional gel electrophoresis system they employed, together with dephosphorylation which can occur during the isolation of ribosomes.

Extensive turnover of ribosomes synthesized in both growth and developmental stages is observed during differentiation of *D. discoideum* [30]. Although only half of the ribosomes present during differentiation are found in polysomes, the same proportion of vegetative and developmental ribosomes are engaged in protein synthesis at any given time during the development of *D. discoideum* [30], suggesting that ribosomes synthesized during differentiation are not essential for translation of developmentally regulated mRNAs. Most of the 5000 unique polyadenylated RNA sequences from growing amoebae continue to be present throughout development [31], whereas about 3000 new polyadenylated RNA sequences, or aggregation-dependent mRNAs, appear in the cytoplasm of developing cells only after the formation of tight cell–cell aggregates. Whether phosphorylated ribosomes play some role in the translation of these late mRNAs, for example by favoring recruitment of mRNAs into polysomes [10], requires further study.

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