JOURNAL OF BACTERIOLOGY, Jan. 1980, p. 20-25
0021-9193/80/01-0020/06$02.00/0

Vol. 141, No. 1

Physiological Control of Phosphorylation of Ribosomal Protein S6 in *Mucor racemosus*

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The level of phosphorylation of ribosomal protein S6 increased with accelerating rates of growth and protein synthesis in *Mucor racemosus*. Lowered levels of phosphorylation were seen under conditions of metabolic shift-down or the onset of stationary phase, and no phosphorylation was detected in sporangiospores. Changing metabolic states, changing intracellular levels of adenosine triphosphatase, and the level of phosphorylation of protein S6 were correlated in *M. racemosus*.

There is evidence for the in vivo modification of ribosomal proteins via acetylation (15) and methylation (4, 5), although their patterns of specificity and their significance as a source of functional heterogeneity is not yet clear. At the present time, phosphorylation of ribosomal proteins represents the most thoroughly characterized form of in vivo modification. Early studies showed that a number of mammalian cells incorporate ^{32}P into ribosome-associated proteins in vivo (2, 6, 11, 21). Gressner and Wool were the first to show that a ribosomal protein defined by two-dimensional polyacrylamide gel methods was phosphorylated in vivo (7). A single protein of the small subunit of rat liver ribosomes, protein S6, incorporated ^{32}P in vivo both in normal tissue and, to a greater extent, in regenerating liver tissue (7). A protein analogous to S6 with a molecular weight of about 31,000 has been found in mouse acites cells (18), baby hamster kidney fibroblasts (15), rabbit reticulocytes (20), HeLa cells (12), and *Saccharomyces* (22). In many cases additional phosphoproteins were observed in each subunit, but S6 was always the major phosphoprotein.

A number of conditions have been shown to change the steady-state extent of S6 phosphorylation in vivo (8, 9, 12, 14). However, it is still not understood what role the phosphorylation of this ribosomal protein plays in either the initiation or the elongation of polypeptide chains.

We have shown that the dimorphic fungus *Mucor racemosus* carried out the in vivo phosphorylation of a ribosomal protein analogous to S6. Although S6 was completely unphosphorylated in dormant spores, it was phosphorylated to varying extents in both the yeast phase and the hyphal phase of the organism (13a). We concluded from those studies that the phosphorylation of S6 was not directly related to morphogenesis. We have continued to study the phosphorylation of this ribosomal protein as a function of the physiological state of the cell. The studies reported here show that the level of phosphorylation of S6 is generally correlated with elevated rates of protein synthesis and with elevated intracellular levels of ATP.

MATERIALS AND METHODS

Organism and culture conditions. *M. racemosus* (*M. lusitanicus*) ATCC 1216B was used in all experiments. Stock cultures were maintained on yeast extract-peptone-glucose (YPG) agar plates, and sporangiospores were transferred to fresh plates at weekly intervals. Cultivation in all experiments was at room temperature (20 to 22°C). Spores were harvested from the plates in distilled water, washed once by centrifugation, and incubated into fresh culture medium. Aerobic hyphae used for ribosome preparations were grown in 400 to 600 ml of YPG medium in a Fernbach flask. After inoculation to 5×10^5 spores per ml, the culture was flushed continuously with 3 ml of air per ml of culture medium per min while the culture was shaken on a rotary shaker. Yeast cells used for ribosome preparations were grown in 1,000 to 1,500 ml of YPG medium and flushed continuously with 0.005 to 0.10 ml of CO_2 per ml of culture medium per min. Shifts of CO_2 yeast cultures to atmospheres of air or nitrogen were carried out by replacing the CO_2 gas with the new gas at a flow rate of 4 ml of gas per ml of culture medium per min. Labeling with ^{32}P was carried out in YPG medium by adding carrier-free $^{32}PO_4^{2-}$ to a final concentration of 50 to 100 μCi/ml. Growth was determined by measuring NaOH-soluble protein. The YPG medium contained 0.3% yeast extract (Difco Laboratories), 1% peptone (Difco), and 2% glucose, pH 4.5. Minimal media were prepared from 0.05% yeast nitrogen base (Difco) without a nitrogen or carbon source. Ammonium-glucose and ammonium-glycerol media were prepared from the yeast nitrogen base by adding 10 mM ammonium chloride and 2% glucose or glycerol. Glutamate-glucose and glutamate-glycerol media were prepared from yeast nitrogen base by adding 10 mM L-glutamate and 2% glucose or glycerol. The media were adjusted to pH 4.5 with sulfuric acid.

Preparations of ribosomes. Cells were harvested from culture media by filtration onto paper disks or by chilling over crushed ice before centrifugation at 4°C. The samples were then frozen in liquid nitrogen without washing and disrupted by grinding for 1 min in a mortar and pestle in liquid nitrogen. The frozen powder was taken up in TKMM buffer (50 mM Tris-hydrochloride, 500 mM KCl, 10 mM magnesium acetate, 12 mM β-mercaptoethanol, pH 7.2). The extract was centrifuged at 30,000 × *g* for 30 min at 4°C. Ribosome subunits were prepared by making the extract 0.1 mM in puromycin-hydrochloride and then incubating it for 20 min at room temperature. From 140 to 180 units of absorbance at 260 nm were layered onto 33 ml of a 10 to 30% linear sucrose gradient in TKMM buffer. Particles were sentimented through the gradient for 7 h at 26,000 rpm in a Beckman SW27 rotor at 10°C. Subunits were recovered from the sucrose by sedimentation at 45,000 rpm in a Beckman 50 Ti rotor. Ribosomal proteins were extracted by suspending the ribosome pellets in TKMM buffer with the magnesium level raised to 100 mM. The extraction was carried out in 66% glacial acetic acid for 1 h at 0°C. The RNA was removed by centrifugation, and the supernatant containing the ribosomal proteins was dialyzed for 24 h at 4°C against 1,000 volumes of 5% acetic acid–0.1% β-mercaptoethanol. These preparations were then concentrated by lyophilization.

Polyacrylamide gel electrophoresis. Ribosomal proteins were separated by two-dimensional polyacrylamide gel electrophoresis, using a modification of the procedure of Kaltschmidt and Wittmann (13). Compositions of the first-dimension gel and the tank buffer were those described by Lastick et al. (14), except that the acrylamide concentration of the gel was increased to 6%, the pH of both solutions was raised to 8.8, and 5 M urea was included in the upper reservoir buffer. The first-dimension gels were 12.5 by 0.6 cm, and the second-dimension gels were 14 by 16 by 0.35 cm. Between 200 and 750 μg of ribosomal protein was dissolved in 150 to 400 μl of 10 M urea (ultrapure; Schwarz/Mann)–3% β-mercaptoethanol, and the solution was heated for 10 min at 60°C. Electrophoresis was carried out for 17 h in the first dimension at 3 mA/gel and for 26 h in the second dimension at 25 mA/gel. Gels were stained with 0.2% Coomassie brilliant blue R (Sigma Chemical Co.) in 25% isopropanol–10% acetic acid. Destaining was accomplished by soaking the gel slabs for at least 48 h in several changes of 10% isopropanol–10% acetic acid.

ATP determination. The intracellular content of ATP was determined by collecting 5- to 20-ml culture samples on membrane filters. The filters were immersed in 8% formic acid; after 30 min at 0°C the formic acid was recovered and lyophilized, and the residue was dissolved in 30 mM sodium arsenate at pH 7.4. A modification of the firefly luciferase assay of Stanley and Williams (19) was used for determining ATP. A 2.0-ml reaction mixture was prepared in a glass scintillation vial containing 10 mM magnesium sulfate–20 mM sodium arsenate (pH 7.4) and 0.10 ml of sample or standard ATP in 30 mM sodium arsenate. The reaction was started with 0.04 ml of reconstituted firefly lantern extract (Sigma). The vial was counted immediately in a Beckman LS-250 scintillation counter set at the sample repeat mode for 0.2 min. The counts accumulated in the ^3H channel during the second counting interval were used to prepare an ATP standard curve and to determine the ATP content in sample extracts.

RESULTS

Figure 1A shows the pattern of total ribosomal proteins from a culture of *M. racemosus* grown to late log phase in hyphal morphology. The subunit assignments of each protein were made by analyzing isolated subunits. Figure 1B shows an autoradiogram from similar gels when the culture was grown in the presence of $^{32}PO_4^{2-}$. Proteins adjacent to S6, S10, and L34 were the only labeled proteins consistently recovered from salt-washed ribosomes (13a).

A set of proteins with lowered first-dimension electrophoretic mobility, appearing as a "string" to the left of S6, represent species of S6 which were phosphorylated in vivo (13a). The amount of protein in each species was quantitated by recovering the stained protein from each species of S6 and measuring the extinction of Coomassie brilliant blue. Figure 2 shows that there are four species of protein; the rightmost spot represents the unphosphorylated form and is trailed by three spots of increasing phosphorylation. The data in Fig. 2 are consistent with the conclusion that the phosphate contents of the forms of S6 are zero, one, two, and three phosphates per molecule. This relationship is obtained by dividing each ratio by the ratio obtained for the first phosphorylated form (i.e. 1,320 cpm per unit of absorbance at 590 nm). The recovery of stained material from each spot was used to quantitate the amount of protein in a given phosphorylated form. This distribution was then expressed as the average steady-state number of phosphates per S6 molecule (P_n) (13a). All of the phosphate in the phosphorylated species of S6 was present in the form of phosphoserine (13a).

By using the methods described above, the extent of S6 phosphorylation was measured at various stages of growth. As Fig. 3 shows, the ribosomes of nongrowing cells (i.e., ungerminated spores) were not phosphorylated, with their P_n's approaching zero. The phosphorylation was evident, however, during all phases of vegetative growth, with a maximum P_n occurring during the early stages of germination when virtually all of the S6 in the cells was present in the triphosphorylated form. This pattern of maximal phosphorylation during the early phase of hyphal growth parallels similar maxima in cyclic AMP levels, rates of respiration (17), and rates of [^3H]leucine incorporation (16). Figure 4 shows that the extent of S6 phosphorylation during the outgrowth of yeasts from spores ger-

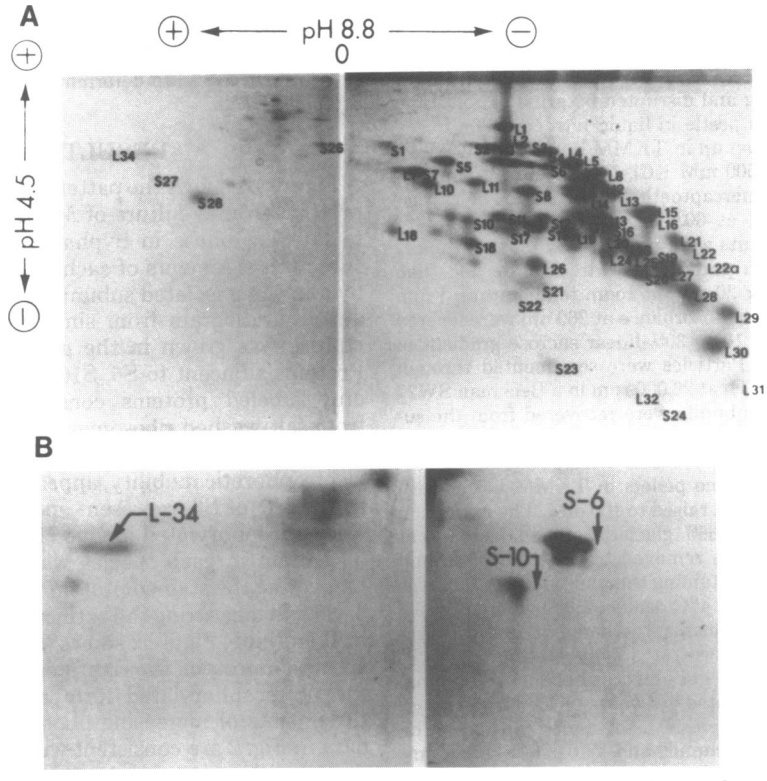

FIG. 1. *(A) Two-dimensional polyacrylamide gel electrophoresis of total ribosomal proteins from M. racemosus. Separate gels were run for the proteins that were acidic (left) and basic (right) at the first-dimension pH of 8.8. Each first-dimension gel was loaded with 700 μg of total ribosomal protein. The protein spots were identified by their locations on the large (L) or small (S) subunit. Two proteins, S25 and L32, were run off the gels in the second dimension. (B) Autoradiogram of two-dimensional gels prepared from proteins isolated from cells grown in $^{32}PO_4^{2-}$. Ten-hour aerobic hyphae were labeled with 100 μCi of ^{32}P per ml in YPG medium. The locations of the spots were determined as in (A).*

minated in 100% CO_2 followed a similar pattern. In this case, however, the maximum level of phosphorylation was about one-half that found in spores germinating to form hyphae (P_n of 1.53 in yeasts compared with 2.74 in hyphae).

The finding that P_n levels rose with the onset of rapid growth and fell with entry of the cells into stationary phase suggested that the phosphorylation of S6 was related to the growth rate. This suggestion was tested by subjecting cells to various cultural changes which either slowed or accelerated growth and protein synthesis. These changes in growth were effected through either carbon or nitrogen shifts-up and shifts-down. Table 1 shows that regardless of the type of limitation, shifts-down resulted in a lowered P_n (i.e., less phosphorylation of S6), whereas shifts-up in growth, including the transfer of cells to fresh YPG medium, resulted in increases in the value of P_n. Table 2 shows that when yeast cells growing in 100% carbon dioxide in YPG medium were shifted to air, there was a steady increase in the level of S6 phosphorylation. In contrast, when hyphal cells growing in air were shifted to CO_2, the P_n fell. Therefore, the increases and decreases in P_n, although apparently correlated with changes in morphology, are more likely the result of changes in the growth rate.

Many of the environmental factors which influence P_n and growth in *M. racemosus* also affect the pattern of energy generation. The peak in P_n during the germination of spores to hyphal cells coincides with a peak in specific respiratory activity (17). Fermentative metabolism and lower P_n's predominate during the later growth of these cells. Low P_n's were observed in cells growing in CO_2 and were correlated with the lowered glycolytic rates evident in these cells (10). The extent of ribosome phosphorylation, therefore, may be positively correlated with the rate of ATP production and negatively correlated with the rate of energy comsumption. The

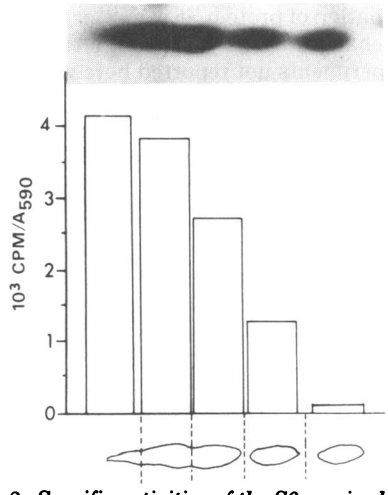

FIG. 2. *Specific activities of the S6 species labeled in vivo with ^{32}P. The species of S6 (shown in the insert in an enlargement of the appropriate portion of a gel) were eluted from the gel. The absorbance of Coomassie brilliant blue at 590 nm (A_{590}) was determined for each sample, and then the radioactivity was measured. Radioactivity in the various spots ranged from 51 cpm for the nonphosphorylated form to 1,620 cpm in the most highly phosphorylated form.*

ATP contents of formic acid extracts of *M. racemosus* were determined by the luciferase assay (19) and were normalized to total cell protein. As Table 3 shows, decreases in ATP levels were observed after the cells were shifted to conditions which also decreased the P_n of protein S6. On the other hand, increases in ATP pool sizes were observed after all of the treatments which increased ribosomal P_n. Overall, these results are consistent with the hypothesis that the level of S6 phosphorylation and the ATP pool sizes are related. However, it is not possible from such correlative data to determine how these effects are related and which might be cause and which might be effect. The data could be explained by the fact that both ATP pool sizes and S6 phosphorylation are related to yet another process. However, neither cyclic AMP nor its dibutyryl analog had any effect on the level of phosphorylation of S6 in either hyphal or yeast cultures. Although the data presented here show that the rates of growth (and protein synthesis) are positively correlated with the phosphorylation of protein S6, the role of nucleotide levels in the control of this event will have to be pursued in more detail.

DISCUSSION

Our experiments on the phosphorylation of ribosomal protein S6 from *M. racemosus* have shown that the phosphorylation event is a dynamic one and undergoes changes throughout the growth cycle of the cells and during changes in metabolism. The relationship between physiological states and the phosphorylation of ribosomal protein S6, which we have shown in *M. racemosus*, has also been found in other cells. For example, the extent of phosphorylation of S6 increases in response to the initiation of growth during rat liver regeneration (7). Similarly, the stimulation of the growth rate of HeLa cells by the addition of fresh medium and serum also induces an increase in the extent of phosphorylation of S6 (14). In *M. racemosus*, changes in culture nutrition which lead to accelerating or decelerating growth rates, as well as changes in the gaseous environment which lead to cellular morphogenesis, all suggest that phosphorylation of S6 is influenced by changes in growth rate and not by the morphology of the cells. The various environmental factors which influence P_n probably influence patterns of energy generation and levels of metabolic intermediates. Since ATP is the phosphate donor in most protein phosphorylations and intracellular levels of

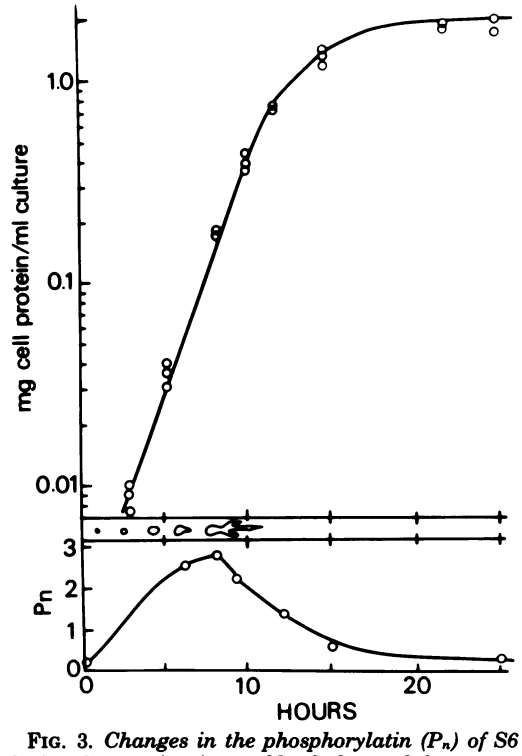

FIG. 3. *Changes in the phosphorylatin (P_n) of S6 during the germination and hyphal growth from sporangiospores. Spores were germinated in air and YPG medium. P_n's were determined by electrophoresis and recovery of stained protein from each species of S6.*

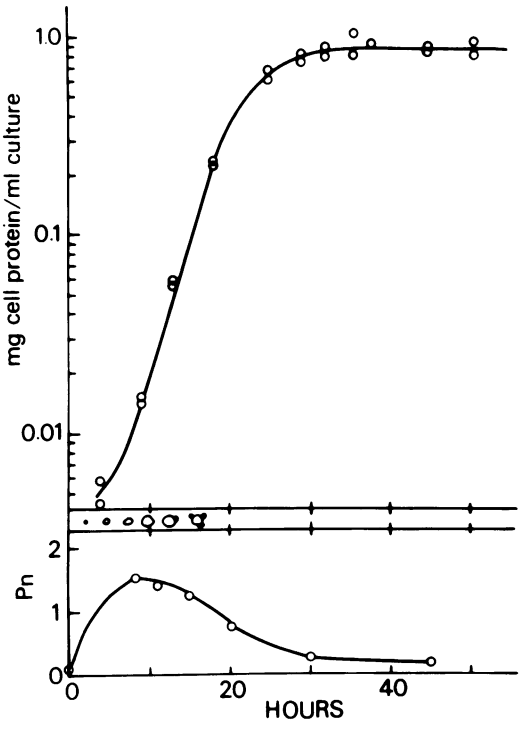

FIG. 4. *Changes in the phosphorylation (P_n) of S6 during the development of yeasts from sporangiospores. Spores were germinated in CO_2 and YPG medium.*

TABLE 1. *Effects of nutritional shifts on the phosphorylation (P_n) of protein S6*[a]

Conditions	P_n
Air-grown hyphae, NH_4^+-glucose medium	1.08
Shifted to NH_4^+-glycerol medium (50 min)	0.32
Shifted to NH_4^+ medium, no carbon source (50 min)	0.22
Air-grown hyphae, glutamate-glycerol medium	0.38
Shifted to glutamate-glucose medium (30 min)	1.35
Air-grown hyphae, YPG medium	1.01
Shifted to fresh YPG medium (30 min)	1.63
Shifted to glutamate-glucose medium (45 min)	0.33
Shifted to NH_4^+-glucose medium (45 min)	0.21

[a] An explanation of P_n and medium compositions are given in the text.

ATP undoubtedly are affected by nutritional shifts, it was reasonable to carry out a preliminary determination of the relationship between ATP pool size and level of ribosomal protein phosphorylation. The important finding of these experiments was that alterations in the phosphorylation of protein S6 were closely correlated with changes in the ATP pool levels (Table 3). In experiments not reported here we found that the addition of various inhibitors of protein synthesis affected P_n. In general, these treatments resulted in an increase in P_n and concomitantly an increase in the levels of ATP. There did not appear to be a correlation between the number

TABLE 2. *Effect of changing morphology on the phosphorylation of protein S6*

Conditions[a]	Time (min)	P_n
20-h CO_2-grown yeasts	0	1.22
Shifted to air	30	1.80
Shifted to air	120	2.37
Shifted to air	240	1.82
12-h air-grown hyphae	0	1.45
Shifted to CO_2[b]	30	0.70
9-h air-grown hyphae	0	2.34
Shifted to CO_2	30	1.30
19-h CO_2-grown yeasts	0	1.35
Shifted to N_2[b,c]	90	2.40

[a] YPG medium was used throughout.
[b] CO_2 and N_2 were used at 100% of the gaseous environment and were provided continuously.
[c] Low-flow N_2, which leads to hyphal development (17).

TABLE 3. *Intracellular ATP levels and phosphorylation of protein S6 during shifts in growth*

Conditions	P_n[a]	ATP level (nmol/mg of protein)
Air-grown hyphae, YPG medium[a]	1.45	12.8
Shifted to CO_2 (30 min)[b]	0.70	8.8
Air-grown hyphae, glutamate-glycerol medium	0.38	18.6
Shifted to glutamate-glucose medium (30 min)	1.35	23.2
CO_2-grown yeasts, YPG medium	1.22	6.9
Shifted to air (120 min)	2.37	15.0
CO_2-grown yeasts, YPG medium	1.22	7.2
Shifted to air (120 min)	2.37	15.0
Shifted to air (240 min)		
Shifted to air (360 min)	1.82	10.4
		4.6

[a] The description of P_n and the compositions of the media are given in the text.
[b] When changes of gaseous atmosphere (e.g., air to CO_2) were made, the medium remained the same.

of ribosomes involved in polysomes and the level of protein S6 phosphorylation. We have reported (13a) that there was no difference in the P_n's of protein S6 derived from ribosome subunits, monosomes, and polysomes.

Although the data reported here add to our knowledge of the metabolic control of the phosphorylation of ribosomal protein S6, it is not yet clear what role this phosphorylation plays in ribosome function. However, it is possible to prepare ribosome subunits of different and known degrees of phosphorylation of this protein, making it possible to apply direct in vitro tests of the role of ribosomal protein phosphorylation in messenger selection, initiation, elongation, and termination of protein synthesis. The appearance of this protein and its specific derivatization in eucaryotes as diverse as *Mucor* and humans are consistent with a functional role for the phosphorylation. It is entirely possible that the phosphorylation of ribosomal protein S6 plays a role in governing the rate of peptide bond formation at the level of the ribosome. This rate has been shown to change over a wide range in eucaryotic cells (1, 3, 16), whereas the rate of protein synthesis in procaryotic cells may be governed largely at the level of transcription.

ACKNOWLEDGMENTS

We thank Kathleen Dimmitt for technical assistance in a portion of this study. We also thank our colleagues P. Borgia, J. Paznokas, and M. Orlowski, for stimulating and critical discussions.

This work was supported in part by Public Health Service grant GM 23999 from the National Institute of General Medical Sciences.

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