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CONTROL OF RIBOSOMAL PROTEIN PHOSPHORYLATION IN HeLa CELLS

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SUMMARY: The effects of a large series of hormones, cyclic nucleotides and metabolic inhibitors on phosphorylation of ribosomal protein S6 in HeLa cells suggest that at least two metabolic pathways are involved. One responds to insulin and epidermal growth factor; the other responds to adenosine 3', 5'-cyclic monophosphate. Some phosphodiesterase inhibitors can suppress the phosphorylation of S6 that ordinarily is stimulated by insulin.

Previous reports from this laboratory have shown that serum or insulin, together with fresh medium, strongly stimulate the phosphorylation of ribosomal protein S6 in spinner cultures of HeLa cells (1, 2). A similar response has been reported for quiescent cultures of 3T3 cells given serum (3) and for 3T3-L1 pre-adipocytes given insulin (4). Glucagon or cAMP[1] can stimulate the phosphorylation of rat liver S6 markedly (5). By contrast, cAMP leads to at most a moderate increase in the phosphorylation of HeLa S6, as judged by the electrophoretic mobility of the protein (2). Here we summarize data that can be interpreted to imply the existence of two metabolic pathways that lead to the phosphorylation of protein S6.

EXPERIMENTAL PROCEDURES

HeLa S3 cells were grown in spinner culture to approximately 7 x 10^5/ml. The cells were then harvested by centrifugation (200 g, 5 min), and resuspended in media containing the agents indicated. After 1 to 2 hr at 37°, the cells were poured over crushed phosphate buffered saline, and harvested by centrifugation. In some cases, ribosomes were isolated and ribosomal proteins were prepared as described (6). In most cases, total cell protein was extracted (2). Approximately 0.1 gm (wet weight) of HeLa cells, sufficient

[1]Abbreviations: cAMP, adenosine 3',5'-monophosphate; cGMP, guanosine 3', 5'-monophosphate; IBMX, 3-isobutyl, 1-methyl-xanthine, RO 20-1724, 3-butoxy-4-methoxy, 4-(3, 4-dialkoxybenzyl)-2-imidazolidinone (16); EGF, epidermal growth factor.

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for two gel analyses of ribosomal proteins, was treated as follows, with vigorous mixing after each addition: 0.1 ml 10 M urea was added, followed by 0.02 ml 1 M $MgCl_2$, 0.24 ml glacial acetic acid, then 0.24 ml of a mixture of H_2O, 1 M $MgCl_2$ and glacial acetic acid in the same proportions (10:2:24). The extract was centrifuged (10,000g, 10 min), the pellet was re-extracted with 0.3 ml of the H_2O:$MgCl_2$:acetic acid mixture, then the supernatants were combined, clarified by centrifugation (10,000g, 10 min), divided into halves and lyophilized. Ribosomal protein or total cell protein was subjected to two-dimensional polyacrylamide gel electrophoresis by the techniques previously described (2, 6). Gels were stained with Coomassie Blue R250 (Schwartz-Mann), and the level of phosphorylation in protein S6 was estimated from the amount of stain in the various positions that correspond to phosphorylated derivatives of S6 (1, 2, 7, 8). The effect of a given agent on S6 phosphorylation was always evaluated relative to the extent of S6 phosphorylation observed in a control preparation from the same cell culture.

RESULTS AND DISCUSSION

We have tested a large variety of agents for their effects on ribosomal protein S6 phosphorylation in HeLa cells. The effect of each agent was qualitatively evaluated by visual inspection of two-dimensional gel patterns that display ribosomal proteins (see Experimental Procedures). The position of protein S6 on two-dimensional gels has been previously shown to vary with the extent of phosphorylation; the most anodally migrating spot may correspond to S6 containing 5 or 6 phosphorus atoms per molecule (7, 8). Figure 1 shows examples of the S6 spot when the protein is virtually non-phosphorylated (panel b), when phosphorylation has been stimulated to a moderate extent

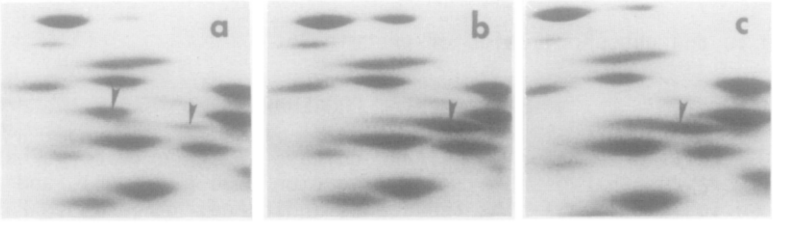

Figure 1. S6 migration on two-dimensional polyacrylamide gels

HeLa S3 spinner culture cells (7 x 10^5/ml) were collected by centrifugation and resuspended in MEM and (a) insulin (5 x 10^{-6}M), (b) insulin (5 x 10^{-6}M) plus IBMX (10^{-3}M), or (c) dibutyryl cAMP (10^{-3}M) plus IBMX (10^{-3}M). The cells were harvested after 1 hr at 37° and total cell protein was subjected to two-dimensional gel electrophoresis. Only the S6 region is shown. Arrows indicate S6 in the several positions that it can occupy depending upon the extent of phosphorylation.

TABLE 1

Effect of Various Agents on Protein S6 Phosphorylation

Agent	Medium	Effect on S6 Phosphorylation
none	Earle's salts	no effect
none	MEM	zero to moderate stimulation
10% calf serum	MEM	strong stimulation
insulin, 5×10^{-8}M	MEM	strong stimulation
insulin, 5×10^{-8}M	Earle's salts	zero to moderate stimulation
epidermal growth factor, 2×10^{-8}M	MEM	moderate stimulation
fibroblast growth factor, 2×10^{-9}M	MEM	no effect
dibutyryl cAMP, 10^{-3}M	MEM	slight stimulation
dibutyryl cGMP, 10^{-3}M	MEM	no effect
glucagon, $\leq 5 \times 10^{-6}$M	MEM	slight to moderate stimulation
cholera toxin, 0.1 μg/ml	MEM	slight stimulation
epinephrine, 5×10^{-6}M	MEM	no effect
IBMX, 10^{-3}M	MEM	no effect
adenine or guanine, 10^{-3}M	MEM	no effect
RO 20-1724, 10^{-3}M	MEM	no effect
prostaglandins E_1 or $F_{2\alpha}$, 5×10^{-6}M	MEM	no effect
cycloheximide, 2×10^{-4}M	MEM	moderate stimulation
puromycin, 2×10^{-4}M	MEM	slight stimulation

In all cases, dense suspension cultures of HeLa cells (6-8 x 10^5 cells/ml) were centrifuged and the cells were resuspended in Minimum Essential Medium (MEM) or Earle's salts, without serum. The indicated agents were added, the cells were harvested 1 or 2 hours later, and the extent of S6 phosphorylation was estimated by the position of the S6 spot(s) on two-dimensional polyacrylamide gels (1, 2, 6). Except for the first two lines, "stimulation" refers to phosphorylation greater than that caused by the medium alone.

(panel c) and when phosphorylation has been strongly stimulated, resulting in most of the protein being maximally shifted in the anodal direction (panel a).

Table 1, which summarizes these studies, supports the following conclusions. (A) Insulin, epidermal growth factor and serum are active stimulators of S6 phosphorylation. Insulin requires amino acids for maximum

effect (2), but EGF will produce maximal phosphorylation of S6 in MRC5 cells (data not shown) even when the cells are suspended in Earle's salts. (B) Dibutyryl cAMP and some agents that should raise the intracellular cAMP concentration (cholera toxin and glucagon) produce a slight stimulation of S6 phosphorylation, but others (epinephrine and prostaglandin E_1) have no detectable effect; nor do purines, nor the phosphodiesterase inhibitors IBMX, RO20-1724, or theophylline (2). Dibutyryl cGMP and prostaglandin $F_{2\alpha}$ have no effect. We emphasize that the term "no effect" in this context means no visible shift of the S6 spot toward the anode in a two-dimensional gel pattern (1). It is quite possible that any or all of these agents raise the level of S6 phosphorylation slightly above the control level, or change the rate of turnover of phosphate, or have other effects which we might not detect. (C) Actinomycin D, a general inhibitor of RNA synthesis, does not by itself stimulate phsophorylation of S6 (not shown), nor does it interfere with phosphorylation stimulated by insulin (Table 2). (D) Protein synthesis is not required for phosphorylation of S6. It is notable that an inhibitor which stabilizes polysomes (cycloheximide) and an inhibitor that leads to polysome disassembly (puromycin) both stimulate S6 phosphorylation in rat liver (9) and in HeLa cells (Table 1). We have also observed that cycloheximide and puromycin reduce the net rate of dephosphorylation of S6; i.e., the rate at which the most anodal form of S6 on a two-dimensional gel disappears after insulin stimulation (unpublished results).

The data in Table 1 suggest that there may be two pathways that lead to phosphorylation of S6; one responds to agents that stimulate cell growth and division (e.g., insulin and EGF); and the other responds to cAMP. This hypothesis is currently being tested by analysis of the tryptic peptides phosphorylated in response to insulin and the peptides phosphorylated in response to cAMP (Lastick, S.M. and McConkey, E.H., unpublished).

If insulin and cAMP lead to phosphorylation of S6 by different pathways, the two pathways might be independent, cooperative, or antagonistic. The

TABLE 2
Effect of Various Agents on S6 Phosphorylation in the Presence of Insulin and Minimum Essential Medium

Agent	Effect on insulin-responsive S6 phosphorylation
dibutyryl cAMP, 10^{-3}M	no effect
dibutyryl cGMP, 10^{-3}M	no effect
IBMX, 10^{-3}	strongly suppressed
guanine or guanosine, 10^{-3}M	moderately suppressed
adenine or adenosine, 10^{-3}M	moderately suppressed
pyrimidines, 10^{-3}M	no effect
isoproterenol, 10^{-3}M	no effect
cholera toxin, 0.1 µg/ml	no effect
RO 20-1724, 1 mM	moderately suppressed
caffeine, 1 mM	moderately suppressed
actinomycin D, 4 X 10^{-6}M	no effect

In all cases, dense suspension cultures of HeLa cells (6 - 8 X 10^5 cells/ml) were centrifuged and the cells were resuspended in Minimum Essential Medium without serum, plus insulin at 5 X 10^{-8}M. The indicated agents were added, the cells were harvested 1 or 2 hours later, and the extent of S6 phosphorylation was estimated by the position of the S6 spot on two-dimensional polyacrylamide gels. In this case, "no effect" means that maximal phosphorylation occurred.

experiments summarized in Table 2 address this question. We find that several phosphodiesterase inhibitors prevent the stimulation of S6 phosphorylation that usually occurs in response to insulin and fresh medium. This could be interpreted to mean that elevation of the intracellular cAMP level antagonizes the insulin response; however, isoproterenol and cholera toxin should also raise the intracellular cAMP (10, 11) and these agents have no effect on insulin-responsive S6 phosphorylation. Cholera toxin is apparently active in this system because it stimulates S6 phosphorylation to a small, but unmistakeable, extent when used without insulin (Table 1), isoproterenol has given ambiguous results in our hands, although it is known that HeLa cells can respond to catecholamines by increasing adenylate cyclase activity (12). Neither dibutyryl cAMP nor dibutyryl cGMP suppresses the phosphorylation of S6 in response to insulin and fresh medium (Table 2).

Further evidence for the existence of separate insulin-responsive and cAMP-responsive pathways for the phosphorylation of S6 comes from the fact that IBMX does not suppress the moderate level of phosphorylation stimulated by dibutyryl cAMP (Figure 1c). We have also observed that IBMX can enhance the phosphorylation stimulated by cholera toxin (data not shown).

Several interpretations of these data are possible. For example, it may be that the phosphodiesterase inhibitors have an effect unrelated to phosphodiesterases; e.g., they might activate a phosphatase that removes phosphate from S6. The analogy with some enzymes of glycogen metabolism is pertinent (13). An alternative possibility is that IBMX, purines, caffeine and RO 20-1724 directly or indirectly inhibit a kinase that phosphorylates S6 in response to growth factors (14, 15). Finally, it may be that the phosphodiesterase inhibitors suppress insulin-dependent phosphorylation of S6 via a phosphodiesterase that hydrolyzes some compound other than cAMP or cGMP. Experiments to test these possibilities are in progress.

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