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Ordered phosphorylation of 40S ribosomal protein S6 after serum stimulation of quiescent 3T3 cells

(two-dimensional gel electrophoresis/thin-layer analysis/phosphoamino acid/tryptic phosphopeptide)

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ABSTRACT **The amino acids and tryptic peptides that become phosphorylated in 40S ribosomal protein S6 after serum stimulation of quiescent 3T3 cells were examined by two-dimensional thin-layer electrophoresis. In the maximally phosphorylated form of the protein, most of the phosphate was incorporated into serine and a small amount, into threonine. Digestion of this form of the protein with trypsin revealed 10 major phosphopeptides. All 10 contained phosphoserine and 2 of the 10 also contained phosphothreonine. Next, the five forms of increasingly phosphorylated S6 were individually separated on two-dimensional polyacrylamide gels or total S6 was isolated from cells that were stimulated for only a short time and their phosphotryptic maps were analyzed. The results showed that, as larger amounts of phosphate were added to S6, the phosphopeptides appeared in a specific order.**

Activation of growth in a number of biological systems is closely associated with multiple phosphorylation of 40S ribosomal protein S6 (1–9). In cultures of quiescent animal cells induced to proliferate by serum, the increase in S6 phosphorylation is temporally correlated with a 2- to 3-fold increase in the rate of protein synthesis (9, 10). In 3T3 cells, the processes respond in parallel to increasing concentrations of serum and epidermal growth factor or to saturating concentrations of the growth factor together with prostaglandin $F_{2\alpha}$ and insulin (11). The increase in phosphorylation is not altered when protein synthesis is completely blocked by cycloheximide. However, complete or partial inhibition of S6 phosphorylation by methylxanthines leads to a corresponding inhibition of protein synthesis (12). These findings are consistent with recent results showing that 40S ribosomes containing the most highly phosphorylated forms of S6 have an apparent selective advantage in entering polysomes (11, 13), suggesting that phosphorylation of S6 is involved in stimulating protein synthesis by facilitating a specific step(s) in the initiation process.

Little is known about the mechanisms that control the overall state of S6 phosphorylation. It has been inferred from the electrophoretic shift on two-dimensional polyacrylamide gels that 5 mol of phosphate are incorporated into the maximally phosphorylated form of the protein (1, 7, 11, 12). *In vitro*, cAMP-dependent kinase incorporates 2 mol of phosphate into S6 (14), and recently the sequences at the two sites have been determined (15). However, it has been argued that these sites are different from those induced by insulin (16, 17). To identify the biochemical events and the enzymes that regulate the level of S6 phosphorylation after the activation of growth, it will first be necessary to carefully characterize the *in vivo* sites of phosphorylation.

We describe here a detailed analysis of the S6 amino acids and the tryptic peptides that become phosphorylated after serum stimulation of quiescent 3T3 cells. The results show that S6 contains both phosphoserine and phosphothreonine, that there are 10 major tryptic phosphopeptides, and that phosphate is added to these peptides in a specific order.

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MATERIALS AND METHODS

Cell Culture and Labeling. Swiss mouse 3T3 cells were grown and maintained as described (11). Cells were judged quiescent 7 to 8 days after seeding when no mitoses were observed. To radioactively label ribosomes to equilibrium with [^{35}S]methionine, 15 μCi of [^{35}S]methionine (1 Ci = 37 GBq) was added to cultures on the 4th day after seeding immediately after refeeding the cells (11). To label cells to high specific activity with $^{32}PO_4$, quiescent cells grown on 15-cm plates were first rinsed with two 10-ml portions of phosphate-free Dulbecco's modified Eagle's medium and then a second 15-ml portion was placed on each culture and $^{32}PO_4$ was added at 0.3–0.5 mCi/ml. Next, the appropriate concentration of fetal calf serum, which had been dialyzed against 10 mM *N*-tris(hydroxymethyl)methylglycine (tricine)-buffered saline (pH 7.4), was added to each culture.

Isolation of ^{32}P-Labeled S6 by Two-Dimensional Gel Electrophoresis. Total cytoplasmic ribosomal proteins from ^{32}P-labeled cells were isolated and separated on two-dimensional polyacrylamide gels as described (11). The gels were stained and autoradiographed, and the S6 derivatives were excised and, together with 100 μg of Phosvitin (Sigma), placed in the elution chamber of an ISCO electrophoretic sample concentrator. Elution of S6 was more than 90% complete after 15 hr at 3 W in 0.1% $NaDodSO_4$/0.1 M $(NH_4)HCO_3$, pH 8.3. After completion of extraction, the 200-μl sample was collected, the sample well was washed with two 1-ml portions of H_2O, and the washings were pooled with the sample. This sample was lyophilized overnight, washed three times at 4°C with 3-ml portions of acetone/H_2O (9:1) to remove $NaDodSO_4$ and Coomassie blue stain, and then relyophilized. The "fluffy" pellet was dissolved in 0.5 ml of performic acid and oxidized for 2 hr. The oxidized material was diluted to 3 ml with H_2O, lyophilized overnight, and either used immediately as described below or stored at −20°C.

Phosphoamino Acid Analysis. The oxidized S6 pellet was taken up in 6 M HCl containing 5 μg each of phosphoserine (Sigma), phosphothreonine (Sigma), and phosphotyrosine (prepared as described in ref. 18). The sample was then incubated under nitrogen at 110°C, lyophilized, and resuspended in 20 μl of H_2O. The resuspended hydrolysate was applied to a 20 × 20 cm precoated cellulose thin-layer plate (Merck) and the phosphoamino acids were separated as described by Hunter and Sefton (19).

Phosphopeptide Analysis. To digest S6, it was incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington) (1 μg of trypsin/4 μg of protein) in 500

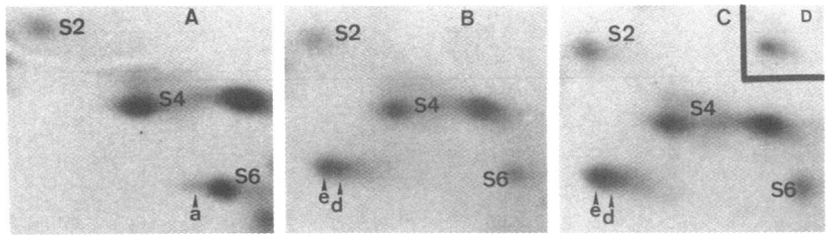

FIG. 1. Quantitation of the extent of S6 phosphorylation by two-dimensional polyacrylamide gel electrophoresis. Ribosomal proteins labeled with [^{35}S]methionine from quiescent cultures (*A*) or from quiescent cultures stimulated with 10% dialyzed serum for 120 min in Dulbecco's modified Eagle's (DME) medium (*B*) or in phosphate-free DME medium (*C*) were extracted, subjected to two-dimensional polyacrylamide gel electrophoresis, and analyzed by fluorography. (*D*) Autoradiogram of a two-dimensional polyacrylamide gel of ribosomal proteins derived from quiescent cells stimulated with 10% dialyzed serum in phosphate-free DME medium containing $^{32}PO_4$.

μl of 0.1 M $(NH_4)HCO_3$ (pH 8.3) at room temperature for 18 hr. Then, a second aliquot, containing the same amount of trypsin, was added and digestion was continued for 7 hr. The digested sample was diluted to 3 ml with H_2O, lyophilized, redissolved in 3 ml of H_2O, and relyophilized. This cycle was repeated three times to eliminate all traces of $(NH_4)HCO_3$. Next, the sample was dissolved in 200 μl of H_2O and transferred to a 1.8-ml Eppendorf tube, the initial tube was washed with a second 200 μl of H_2O, and the combined sample and washings were centrifuged at 12,000 rpm for 10 min at room temperature. The supernatant was lyophilized, suspended in 20 μl of H_2O, applied to a precoated cellulose thin-layer plate (Merck), and electrophoresed in the first dimension toward the cathode at 1.5 kV for 1 hr in acetic acid/formic acid/H_2O, pH 1.5 (3:1:16). The plate was dried for 2 hr with a fan; electrophoresis in the second dimension was toward the cathode at 1 kV for 2 hr in acetic acid/pyridine/H_2O, pH 3.5 (10:1:89). The plates were dried and autoradiographed at −80°C using intensifying screens (Dupont Cronex) and Kodak XR5 x-ray film.

RESULTS

Serum Stimulation in Phosphate-Free Medium. To label ribosomal protein S6 to high specific activity, quiescent cells were stimulated with 10% dialyzed serum in phosphate-free medium. However, because phosphate deprivation leads to inhibition of cell growth (20), it was first necessary to determine whether these conditions also altered the serum-stimulated increase in S6 phosphorylation and protein synthesis. Quiescent cells, either previously labeled to equilibrium with [^{35}S]-methionine or parallel unlabeled cultures, were stimulated with 10% dialyzed serum in media containing either the normal amount of phosphate (1 mM) or no phosphate. S6 phosphorylation was measured by altered electrophoretic mobility on two-dimensional polyacrylamide gels and protein synthesis was measured by the number of ribosomes as polysomes. The [^{35}S]methionine fluorograms (Fig. 1A) showed that most of the S6 in quiescent cells appeared at the position for the native (unphosphorylated) protein, with a small amount at the position for the first phosphoderivative, S6a. After addition of serum, the majority of S6 electrophoresed in the positions of the maximally phosphorylated derivatives S6d and e (Fig. 1*B*). This increase was unaffected by the absence of phosphate (Fig. 1*C*). Furthermore, after addition of serum, the number of ribosomes as polysomes increased 2- to 3-fold and, as with S6 phosphorylation, the absence of phosphate had no effect on this increase (Table 1). Therefore, removal of phosphate does not appear to affect protein synthesis or S6 phosphorylation during the proposed labeling period.

Table 1. Polysome formation

Sample	% ribosomes as polysomes
Quiescent	28
Serum stimulated	64
Serum stimulated without phosphate	62

Quiescent cells or quiescent cells that had been stimulated with 10% dialyzed serum for 2 hr in the presence or absence of phosphate were harvested and the number of ribosomes as polysomes was determined as described (21).

Analysis of Phosphoamino Acids. Under the phosphate-free conditions described above, quiescent cells were stimulated with 10% dialyzed serum in the presence of $^{32}PO_4$. After 2 hr, the cells were harvested and the ribosomal proteins were separated by two-dimensional polyacrylamide gel electrophoresis and autoradiographed (Fig. 1*D*). Then, S6 was eluted from the gel, and a portion of the eluate was hydrolyzed with HCl in the presence of phosphoamino acid standards and analyzed by two-dimensional thin-layer electrophoresis. By superimposing the autoradiogram of the thin-layer plate (Fig. 2) over the ninhydrin staining pattern, we found that $^{32}P_i$ coelectrophoresed with phosphoserine and phosphothreonine. However, of the total $^{32}P_i$ present, most was found to migrate with free PO_4. In addition, a small amount ran as four unique spots near the origin. When these spots were eluted from the plate and rehydrolyzed, they were converted to phosphoserine, phosphothreonine, or

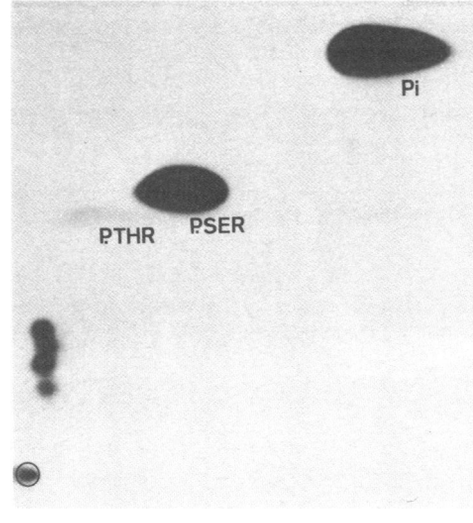

FIG. 2. Two-dimensional analysis of phosphoamino acids. ^{32}P-Labeled S6 was eluted from a two-dimensional polyacrylamide gel (Fig. 1*D*), hydrolyzed for 2 hr with HCl, and analyzed by two-dimensional thin-layer electrophoresis. P.THR, phosphothreonine; P.SER, phosphoserine.

Table 2. Amount of $^{32}P_i$ present in products of hydrolysis

Product(s)	Cherenkov radiation*
Origin	26
Undigested peptides	72
Phosphothreonine	21
Phosphoserine	904
P_i	1,576

Each product of acid hydrolysis in Fig. 2 was scraped from the thin-layer plate and eluted, and the amount of Cherenkov radiation was determined.

* In each case, an area corresponding in size to each product of hydrolysis, but containing no radioactivity as judged by autoradiography, was processed as above and the amount of Cherenkov radiation present was subtracted as background.

free PO_4, suggesting that they represent incompletely hydrolyzed protein (data not shown). To quantitate the absolute amount of $^{32}P_i$ present in each species, the spots were eluted from the plate and their radioactivity was determined (Table 2). Of the total $^{32}P_i$ present in amino acids, 98% comigrated with phosphoserine and 2% comigrated with phosphothreonine. However, the total amount of $^{32}P_i$ present in phosphoamino acids was only 36% of the total eluted from the plate, with 61% present as free PO_4. The rapid release of $^{32}PO_4$ from S6 relative to other ribosomal phosphoproteins is in agreement with the findings of others (1, 22).

Analysis of Tryptic Peptides. To determine the number of sites that become phosphorylated after serum induction, the remaining portion of the maximally phosphorylated S6 (Fig. 1*D*) was digested exhaustively with trypsin and analyzed by two-dimensional thin-layer electrophoresis. Ten major phosphopeptides and several minor ones could be resolved on the autoradiogram of the thin-layer plate (Fig. 3). In addition, in some experiments, it was possible to separate phosphopeptide 5 into two components, a and b (see Fig. 5). To determine the phosphoamino acids present in the individual phosphopeptides, each spot was eluted from the gel, hydrolyzed, and analyzed as described above. The results showed that all phosphopeptides contained phosphoserine and that phosphopeptides 6 and 10 also contained phosphothreonine (Table 3).

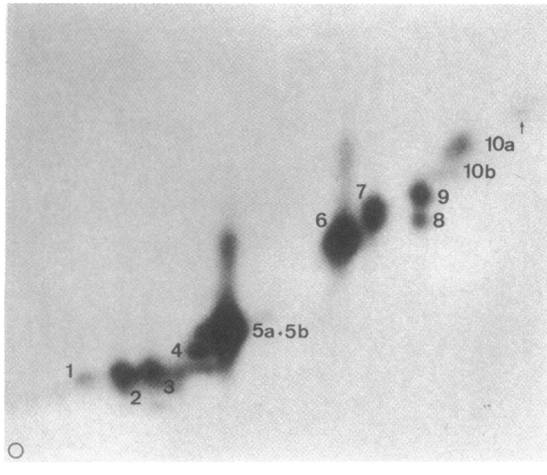

FIG. 3. Two-dimensional analysis of tryptic phosphopeptides. ^{32}P-Labeled S6 was eluted from a two-dimensional polyacrylamide gel (Fig. 1*D*) and digested with trypsin, and the digest was subjected to two-dimensional thin-layer electrophoresis and analyzed by autoradiography. ○, Origin of sample application; ↑, minor phosphopeptide. In the first dimension, electrophoresis was from left to right at pH 1.9 toward the cathode; in the second dimension, it was from bottom to top at pH 3.5 toward the cathode.

Table 3. Phosphoamino acid analysis of tryptic phosphopeptides

Phosphopeptide	Phosphoserine	Phosphothreonine
1	+	−
2	+	−
3	+	−
4	+	−
5a/5b	+	−
6	+	+
7	+	−
8	+	−
9	+	−
10a/10b	+	+

Each of the phosphopeptides in Fig. 3 was scraped from the plate, eluted, hydrolyzed, and analyzed by two-dimensional thin-layer electrophoresis. +, Detected; −, not detected.

Order of Phosphorylation. Because S6 can be separated into five distinct derivatives, depending on the extent to which it is phosphorylated, we asked whether each of the increasingly phosphorylated derivatives contains all the major phosphopeptides or whether instead each derivative is represented by a unique phosphopeptide or set of phosphopeptides. Previously, it has been shown with cells stimulated with 2.5% serum for 2 hr that each of the S6 derivatives except for d and e can be resolved from the others and that all are present in similar amounts (6, 11). Using these conditions, in the presence of $^{32}PO_4$ (Fig. 4), we isolated each derivative of S6, treated it with trypsin and, depending on the extent to which it was phosphorylated, analyzed an appropriate amount of radioactivity from each digest by two-dimensional thin-layer electrophoresis (Fig. 5). The results showed that only phosphopeptides 10a and 10b and two minor components could be detected in derivative S6a (Fig. 5*A*). In derivative S6b, in addition to phosphopeptides 10a and 10b, three new phosphopeptides were present (labeled 7, 8, and 9 in Fig. 5*B*) as well as two minor peptides and a number of weakly labeled spots near the origin (Fig. 5*B*). Derivative S6c is almost identical to S6b except for the addition of two new phosphopeptides, 2 and 4 (Fig. 5*C*). Finally, derivatives S6d and -e together contain all the phosphopeptides present in the preceding derivatives plus phosphopeptides 1, 3, 5a, 5b, and 6 (Fig. 5*D*).

The results described above show that each phosphorylated derivative of S6 contains the set of phosphopeptides present in the immediately preceding less-phosphorylated derivative as well as a unique set of new phosphopeptides. Note also that the phosphopeptides present in any given derivative do not appear to increase in intensity in the next more-highly phosphorylated derivative. These findings imply that, as S6 becomes increasingly phosphorylated, the phosphates are added in a specific

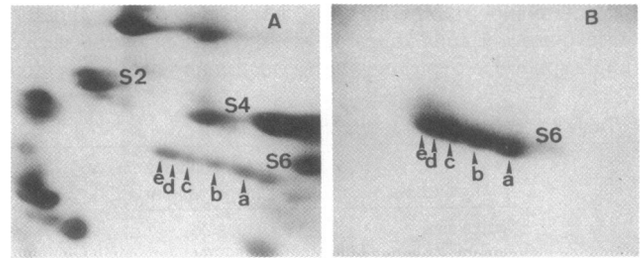

FIG. 4. Partial activation of S6 phosphorylation. Quiescent 3T3 cells (8.0×10^7) were stimulated with 2.5% dialyzed serum in phosphate-free medium containing $^{32}PO_4$ for 2 hr. Ribosomal proteins were extracted and separated by two-dimensional polyacrylamide gel electrophoresis, and the gels were stained with Coomassie blue (*A*) and autoradiographed (*B*).

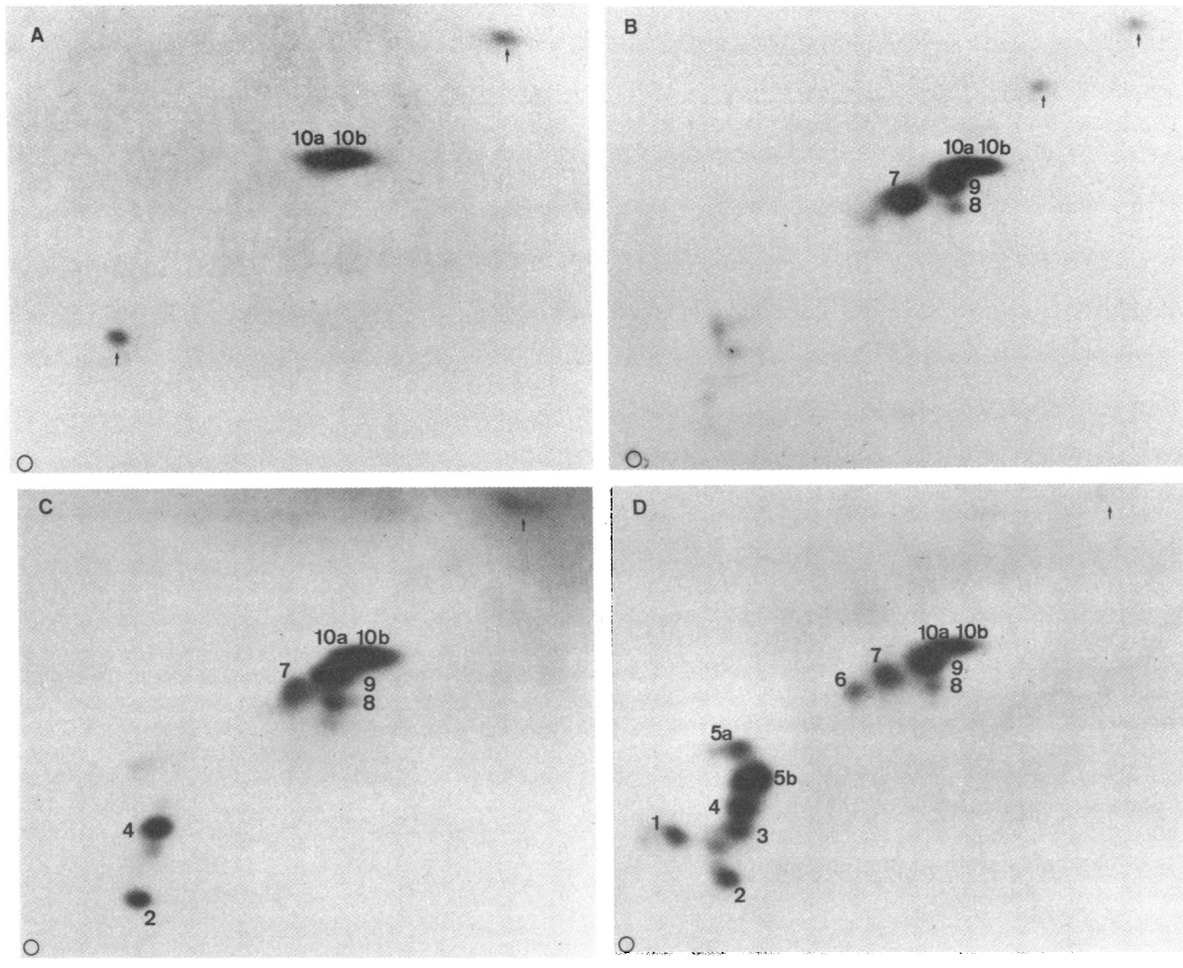

FIG. 5. Tryptic phosphopeptide analysis of S6 derivatives. Each of the S6 derivatives was eluted from a two-dimensional polyacrylamide gel (Fig. 4) and analyzed as in Fig. 3. (*A*) Derivative S6a (1,000 cpm). (*B*) Derivative S6b (2,000 cpm). (*C*) Derivative S6c (3,000 cpm). (*D*) Derivative S6d/e (4,500 cpm).

order. To test this possibility, cells were stimulated with 10% serum in the presence of $^{32}PO_4$ for only 10 min. Total S6 was then isolated on a one-dimensional polyacrylamide gel and the phosphopeptides were analyzed by two-dimensional thin-layer electrophoresis. Under these conditions of stimulation, the most heavily labeled phosphopeptides were 10a and 10b, and these were followed by phosphopeptides 7, 8, and 9, and then by 2 and 4 (Fig. 6). The other phosphopeptides were not detectable at this time. The results from Figs. 3, 5, and 6 taken together argue that the phosphates that are incorporated into S6 after serum induction are added in a specific order.

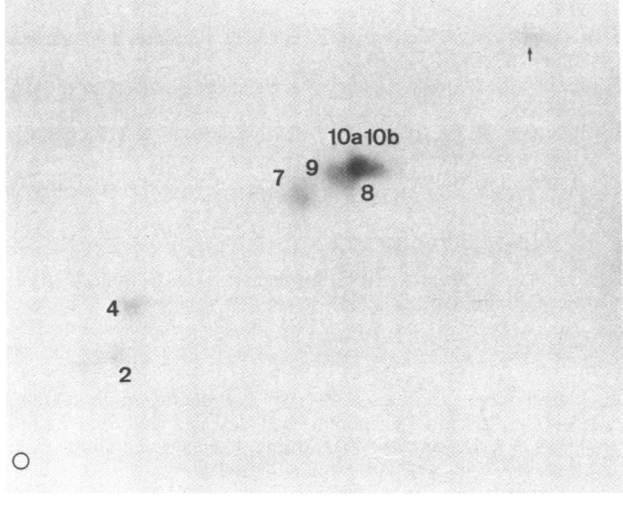

FIG. 6. Autoradiogram of two-dimensional thin-layer electrophoresis analysis of phosphopeptides of total S6 derived from cells stimulated with 10% serum for 10 min. Total S6 was extracted from the first dimension of a two-dimensional polyacrylamide gel and analyzed as described in Fig. 3. ○, Origin of sample application; ↑, minor phosphopeptide.

DISCUSSION

Both phosphoserine and phosphothreonine are detectable in S6 after serum stimulation of resting 3T3 cells (Fig. 2). This finding confirms our preliminary report that S6 isolated from one-dimensional $NaDodSO_4$ gels contains both these phosphoamino acids (23). However, because a large amount of phosphate is lost from S6 during acid hydrolysis (Table 1), the possibility exists that phosphate may be present as some other more labile phosphoamino acid, such as phosphotyrosine (19). To test this possibility, S6 was first digested with trypsin (which led to no measurable release of PO_4) and then either extensively digested with Pronase E or hydrolyzed with HCl for short times (data not shown). Under none of the conditions used were we able to detect any phosphoamino acid other than phosphoserine or phosphothreonine.

The results in Fig. 5 show that each increasingly phosphorylated derivative of S6 is represented by a unique set of phosphopeptides. These data together with the findings shown in

Figs. 3 and 6 show that this phosphate is added to these 10 major phosphopeptides in a specific order. If each of these phosphopeptides represented a unique site of phosphorylation, this would suggest that individual 40S ribosomal subunits contain differentially phosphorylated forms of S6. This is because it is inferred from two-dimensional polyacrylamide gels that only five mol of phosphate can be incorporated into the maximally phosphorylated form of S6 and there is only 1 mol of S6 per 40S subunit (24). A more likely explanation might be that several phosphopeptides contain the same sites of phosphorylation and are simply overlapping sequences of these same peptides generated by partial degradation during isolation or by nonspecific cleavage during trypsin digestion. This last alternative is supported by the fact that each increasingly phosphorylated derivative of the protein (Fig. 4) is represented by a new set of phosphopeptides (Fig. 5), all of which have similar electrophoretic mobilities. The final distinction between these alternatives will require the determination of the sequences of the phosphopeptides. However, it should be noted that this sequence determination will not alter the conclusion drawn from Figs. 3, 5, and 6 that the phosphate is incorporated into S6 in a specific order.

If there are 5 major sites of phosphorylation, or even 10, the small amount of phosphothreonine (2%) found in S6 is surprising. The portion of phosphothreonine might have been expected to be in the order of 10–20%. One possibility is that there is a sixth additional minor site of threonine phosphorylation. However, if this were the case, one might have expected an additional minor derivative more acidic than derivative S6e. We have not observed such a derivative. Another possible explanation is that there is a cross-contaminating phosphoprotein running in the same position as S6. This possibility also seems unlikely because not only would this protein have to coelectrophorese on two-dimensional polyacrylamide gels with two forms of S6 (derivatives S6a and S6d/e) but also its phosphopeptides would have to coelectrophorese together with S6 phosphopeptides (6 and 10) on thin-layer plates. A more intriguing possibility is that there exists within S6 a phosphopeptide that contains a serine and a threonine but that, on any single molecule of S6, only one can be phosphorylated. Such a hypothesis would also be consistent with the 10–15% or 20–25% phosphothreonine found respectively in phosphopeptides 6 and 10 (data not shown), because a pure phosphopeptide, as described above, containing both amino acids phosphorylated would have to have 50% of each.

Although S6d and -e cannot be completely resolved from each other, it is possible to estimate the amounts of each. For example, there is more of S6e than of S6d in cells stimulated with 10% serum (Fig. 1 *C* and *D*) and less in cells stimulated with 2.5% serum (Fig. 4). Furthermore, the intensity of phosphopeptide 6 appears to change in parallel with the relative amount of S6e present (compare Figs. 3 and 5*D*), suggesting that this site of phosphorylation is confined to derivative e. Since it has recently been shown that derivative S6e is apparently the most efficient of all the S6 derivatives in entering polysomes (11), it may be that phosphopeptide 6 contains a crucial site of phosphorylation involved in increasing the rate of protein synthesis (11). In this regard, it should be noted that phosphopeptide 6 is not detectable 10 min after stimulation (Fig. 6) whereas it is 20 min after stimulation (data not shown) and no change is detected in the rate of protein synthesis until 15–30 min after stimulation (12).

Little is known concerning the S6 kinases; however, several candidates exist (5, 25, 26). Clearly, it will be of interest to know how the *in vitro* and *in vivo* sites of these kinases compare with those in 3T3 cells. In addition, it has recently been shown that different growth factors added separately to quiescent 3T3 cells induce only partial S6 phosphorylation (12). However, when added together, they can induce levels of phosphorylation equal to those of 10% serum (12). The use of the phosphopeptide maps will now make it possible to compare the sites of phosphorylation induced by individual growth factors.

Finally, since phosphates are added to S6 in a specified order, it is possible that the phosphorylation of one site may be dependent on the prior phosphorylation of another site. This possibility will have to be considered in future attempts to isolate and characterize the S6 kinase(s) and phosphatase(s).

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1. Gressner, A. & Wool, I. (1974) *J. Biol. Chem.* **249**, 6917–6925.
2. Anderson, W., Grundholm, A. & Sells, B. (1975) *Biochem. Biophys. Res. Commun.* **82**, 669–676.
3. Decker, S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4112–4115.
4. Ballinger, D. & Hunt, T. (1981) *Dev. Biol.* **87**, 277–285.
5. Nielsen, P. J., Thomas, G. & Maller, J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2937–2941.
6. Nielsen, P. J., Manchester, K. L., Towbin, H., Gordon, J. & Thomas, G. (1982) *J. Biol. Chem.* **257**, 12316–12321.
7. Lastick, S. M., Nielsen, P. J. & McConkey, E. H. (1977) *Mol. Gen. Genet.* **152**, 223–230.
8. Haselbacher, G., Humbel, R. E. & Thomas, G. (1979) *FEBS Lett.* **100**, 185–190.
9. Thomas, G., Siegmann, M. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3952–3956.
10. Duncan, R. & McConkey, E. H. (1982) *Eur. J. Biochem.* **123**, 539–544.
11. Thomas, G., Martin-Pérez, J., Siegmann, M. & Otto, A. (1982) *Cell* **30**, 235–242.
12. Thomas, G., Siegmann, M., Kubler, A. M., Gordon, J. & Jimenez de Asua, L. (1980) *Cell* **19**, 1015–1023.
13. Duncan, R. & McConkey, E. H. (1982) *Eur. J. Biochem.* **123**, 535–538.
14. Del Grande, R. W. & Traugh, J. A. (1982) *Eur. J. Biochem.* **123**, 421–428.
15. Wettenhall, R. E. H. & Cohen, P. (1982) *FEBS Lett.* **140**, 263–269.
16. Lastick, S. M. & McConkey, E. H. (1980) *Biochem. Biophys. Res. Comm.* **95**, 917–923.
17. Lastick, S. M. & McConkey, E. H. (1981) *J. Biol. Chem.* **256**, 583–585.
18. Rothberg, P. G., Harris, T. J. R., Nomoto, A. & Wimmer, E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4868–4872.
19. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1311–1315.
20. Holley, R. W. & Kiernan, J. A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2942–2945.
21. Thomas, G., Siegmann, M., Bowman, P. D. & Gordon, J. (1977) *Exp. Cell Res.* **108**, 253–258.
22. Leader, D. P. & Coia, A. A. (1978) *Biochim. Biophys. Acta* **519**, 224–232.
23. Thomas, G., Siegmann, M., Gordon, J., Jimenez de Asua, L., Martin-Pérez, J. & Nielsen, P. (1981) in *Protein Phosphorylation*, Cold Spring Harbor Conferences on Cell Proliferation, eds. Rosen, O. & Krebs, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 8, pp. 783–799.
24. Howard, G. A., Traugh, J. A., Croser, E. A. & Traut, R. R. (1975) *J. Mol. Biol.* **93**, 391–404.
25. Traugh, J. A. (1981) *Biochem. Actions Horm.* **8**, 167–204.
26. Wu, M. & Gerhart, J. C. (1980) *Dev. Biol.* **79**, 465–477.