Phosphopeptide patterns of the ribosomal protein S6 following stimulation of guineapig parotid glands by secretagogues involving either cAMP or calcium as second messenger

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Stimulation of secretion in exocrine cells is associated with the incorporation of up to 3 to 4 phosphates into the ribosomal protein S6. This occurs with secretagogues involving either cAMP or free calcium as second messenger. An analysis of the phosphorylation pattern of S6 from stimulated guineapig parotid glands reveals 3 phosphopeptides (termed A,B,C). The phosphopeptide pattern was identical for cAMP- or calcium-mediated stimulation, whereas phosphorylation of the S6 protein in vitro with catalytic subunit of cAMP-dependent protein kinase resulted only in the formation of phosphopeptides A and C. Therefore, secretagogue-mediated phosphorylation is not or not exclusively catalyzed by cAMP-dependent protein kinase even when cAMP is the second messenger.

Protein S6 *Phosphopeptide pattern* *Parotidgland, of guineapig* *Secretagogue* *Cyclic AMP* *Ca^{2+}-mediated stimulation*

1. INTRODUCTION

Secretion of exportable proteins from exocrine glands can be stimulated by specific secretagogues involving different second messengers [1]. One of the molecular events in exocrine glands following stimulation with secretatogues is the enhancement of the phosphorylation of specific proteins [2–6]. The only protein the phosphorylation of which is enhanced after cAMP- as well as Ca^{2+}-mediated stimulation is the protein S6 from the small ribosomal subunit [3b,7]. Its phosphorylation is closely related to the secretion of exportable protein [2,3a].

S6 is phosphorylated in a variety of cells in response to many different agents (see [8] for review). In vitro S6 can be phosphorylated by the catalytic subunit of the cAMPdPK [9,10], cGMPdPK [9], a protease-activated protein kinase [11], and a Ca^{2+}-activated protein kinase [3c]. For reticulocytes it has been shown [9] that some of the phosphopeptides derived after phosphorylation in vivo could be phosphorylated in vitro by the catalytic subunit of cAMPdPK. For hepatocytes [12] and HeLa cells [13] it has been shown, that different agonists led to significantly different phosphopeptide patterns of S6.

An analysis of the tryptic phosphopeptide pattern of protein S6 after different types of stimulation of parotid glands was therefore performed in order to get an indication of whether different types of protein kinases might be involved with different second messengers. According to our study, agonists involving cAMP or Ca^{2+} as second messenger led to the same phosphopeptide pattern.

Abbreviations: cAMPdPK, cyclic adenosine monophosphate-dependent protein kinase; cGMPdPK, cyclic guanosine monophosphate-dependent protein kinase; cAMP, cyclic adenosine monophosphate

2. MATERIALS AND METHODS

2.1. *Materials*

Male guineapigs of the Pearlbright White strain (250–300 g body wt) were obtained from Lippische

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Versuchsanstalt (Lage). D,L-Isoproteronol and carbamylcholine were supplied by Sigma. [^{32}P]Orthophosphate was obtained from Amersham Buchler. Trypsin (TPCK-treated) and trypsin (type 1) were purchased from Worthington and Sigma, respectively. DEAE–Trisacryl was obtained from LKB, cellulose thin-layer sheets (MN Cel 300) from Machery and Nagel (Aachen). All other reagents were analytical grade and came from Merck (Darmstadt).

2.2. *Preparation of ^{32}P-labeled ribosomes from the parotid gland and purification of S6 protein by polyacrylamide gel electrophoresis*

Parotid lobules were prepared as in [14]. The tissue was preloaded with [^{32}P]phosphate, incubated and stimulated using 400–500 mg wet wt instead of 100 mg. After addition of isoproterenol or carbamylcholine the incubation was continued for another 20 min and stopped by homogenisation at 4°C in 0.3 M sucrose, 50 mM potassium phosphate (pH 7.0), 2 mM EDTA, 0.2 mM EGTA. A crude ribosomal fraction was prepared by centrifugation after dissolving the microsomal membranes by desoxycholate [7]. Ribosomal proteins were extracted with 67% (v/v) acetic acid for 3–4 h, dialysed against 0.5% acetic acid and lyophilized. Total ribosomal proteins from the parotid were first separated on one-dimensional polyacrylamide gels using the SDS buffer system in [15]. Gels were stained with Coomassie brilliant blue and destained. Under our conditions no other phosphoprotein comigrated with S6 as checked by two-dimensional electrophoresis [16]. After localization by autoradiography the S6 bands were excised from the gel, the protein was eluted [17] and recovered by acetone precipitation after addition of 200 μg ribosomal protein as carrier. Total S6 protein was separated into different phosphorylated species by two-dimensional gel electrophoresis as in [7,18].

2.3. *Tryptic cleavage and fingerprinting of S6 phosphopeptides*

Gel pieces containing ^{32}P-labeled S6 were subjected to tryptic digestion as in [19] with the following modifications: the lyophilized gel pieces were incubated in 0.3 ml of a solution containing 2 μg trypsin and 2.5 μg TPCK in 50 mM ammonium bicarbonate, 5 mM dithioerythritol at 37°C. After 12 h the supernatants were removed, and the remaining peptides eluted with 0.3 ml water at 37°C for another 4 h. The elution was repeated until at least 80% of the radioactivity was eluted. The combined supernatants were again incubated for 3.5 h in the presence of trypsin (2 μg) and TPCK (2 μg). Ammonium bicarbonate can be substituted by triethylammonium bicarbonate without affecting the cleavage pattern.

2.4. *Separation of S6 phosphopeptides*

Tryptic phosphopeptides were separated either by thin-layer chromatography [20] or by ion-exchange chromatography [21]. For fingerprinting the samples were lyophilised and the remaining ammonium bicarbonate was completely removed in a vacuum oven at 60°C for 24 h. The resulting peptides were separated on cellulose thin-layer chromatography sheets as [20] with electrophoresis in 10% (v/v) acetic acid, 1% (v/v) pyridine (pH 3.5) for 70 min at 500 V in the first dimension and chromatography in *n*-butanol/pyridine/acetic acid/water (37.5:25:7.5:30, by vol.) in the second dimension. The dried plates were autoradiographed with Fuji RX film.

For ion-exchange chromatography an aliquot of the incubation mixture was loaded onto a DEAE–Trisacryl column (1 × 30 cm) which was equilibrated with 50 mM triethylammonium bicarbonate. The column was eluted with a linear gradient of 50–300 mM triethylammonium bicarbonate buffer (each 150 ml). Fractions of 1 ml were collected and the radioactivity in each fraction was measured by Cerenkov counting. Recovery from the DEAE–Trisacryl columns was about 90% of the input radioactivity. Individual phosphopeptides eluting from the column were collected, lyophilized and subjected to fingerprinting in order to correlate the phosphopeptides of the elution profiles to the radioactive spots on the fingerprints.

2.5. *Analysis of phosphoamino acids*

The S6 containing portion was excised from the gel and hydrolyzed in 6 N HCl at 100°C for 90 min under N_2. After evaporation of HCl and washing with water the phosphoamino acids were analysed on paper (Whatman 3) by high-voltage electrophoresis [22].

3. RESULTS AND DISCUSSION

The stimulation of guineapig parotid gland lobules with secretagogues enhances the phosphorylation of a 30 kDa protein (fig. 1), after stimulation with isoproterenol (which involves cAMP as second messenger) as well as with carbamylcholine (which involves calcium as second messenger). This corresponds to the situation found in exocrine organs of rat and mouse [2–6]. The 30 kDa protein has been identified as the ribosomal protein S6 from the small ribosomal subunit [3b,7].

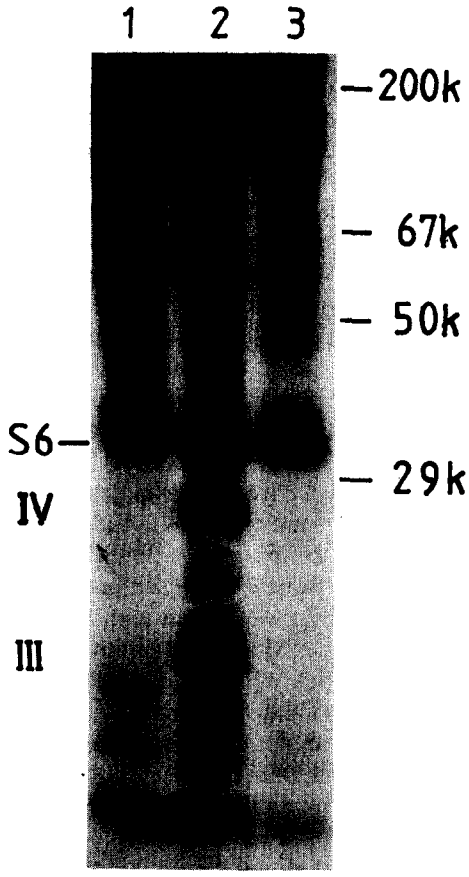
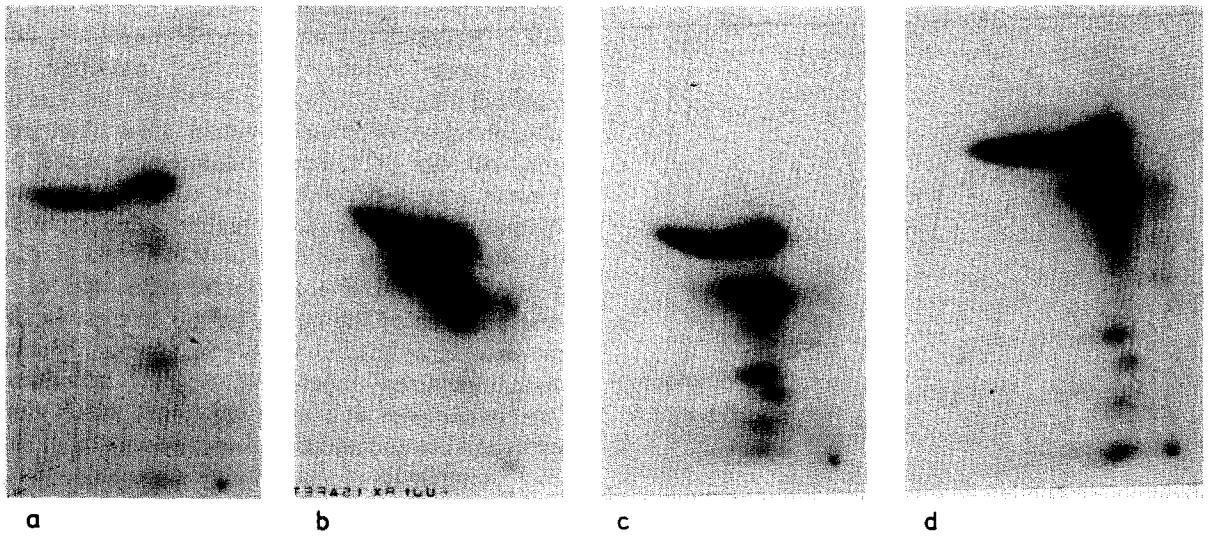


Fig. 1. Separation of phosphoprotein of the parotid gland by SDS–polyacrylamide electrophoresis. Parotid lobules were incubated with [^{32}P]phosphate, stimulated, and after homogenization centrifuged at 10000 × *g* for 5 min and at 100000 × *g* for 60 min. The microsomal pellet was separated by SDS–polyacrylamide electrophoresis. The autoradiograph is shown: (a) control without secretagogue; (b) stimulation with 2×10^{-5} M isoproterenol; (c) stimulation with 10^{-6} M carbamylcholine. Protein III has been described in [2,14], Protein IV is specific for guineapig organs (in preparation).

The fingerprint of tryptic phosphopeptides derived from unfractionated phosphorylated protein S6 after incubation in the absence or presence of secretagogues is shown in fig. 2. Already in the unstimulated state, two phosphopeptides (A,B in fig. 2) can be demonstrated. After stimulation by either isoproterenol or carbamylcholine the phosphorylation of these two phosphopeptides is enhanced and in addition a third phosphopeptide (C in fig. 2) appears. The identity of the 3 phosphopeptides phosphorylated during stimulation by the two types of agonists is shown by the fact that mixing the proteolytic digests from S6 from isoproterenol-stimulated glands with those from carbamylcholine-stimulated glands again results in the same phosphopeptide pattern without showing additional phosphopeptides (fig. 2d). After carbamylcholine treatment sometimes small amounts of additional phosphorylated peptides were found, which apparently represent peptides appearing only when the phosphorylation of S6 reaches a higher degree (see below). The only phosphoamino acid found in S6 from the parotid gland is phosphoserine (not shown).

When the phosphopeptides are separated by anion-exchange chromatography, again the same 3 phosphopeptides are found, irrespective of the secretagogue used (fig. 3). Individual peptides were isolated from the eluates and separated by thin-layer chromatography in order to identify and correlate the phosphopeptides from the elution profiles (fig. 3) and the fingerprints (fig. 2). This comparison showed that the 3 peaks of the column profile correspond from left to right to the phosphopeptides A, B and C, respectively (not shown). The differences in the relative height of the single peaks was not consistent and gave no indication for an agonist-specific difference (note that in fig. 2, phosphopeptide A seems to be preferentially labeled under isoproterenol whereas in fig. 3 this would hold for phosphopeptide C).

Phosphorylated S6 protein was separated into the individual S6 derivatives by two-dimensional polyacrylamide gel electrophoresis as in [7]. In the S6 region of the control cells and of isoproteronol-treated cells two phosphorylated S6 derivatives



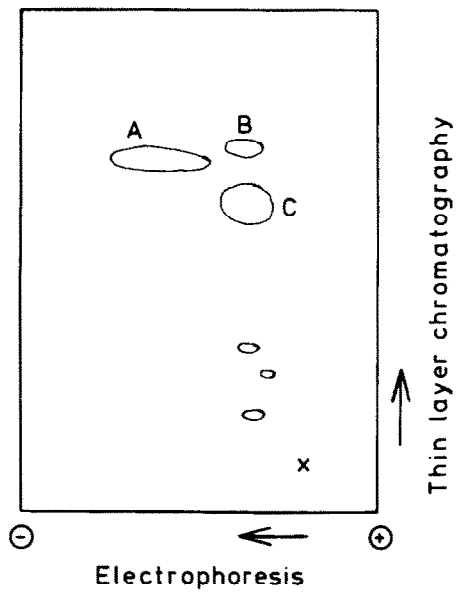


Fig. 2. Phosphopeptides from intracellularly phosphorylated protein S6. The protein band containing S6 (fig. 1) was excised from the gel, digested with trypsin, and the phosphopeptides separated by electrophoresis and thin-layer chromatography on cellulose thin-layer sheets. The autoradiographs are shown: (a) control incubation; (b) stimulation with 2×10^{-5} isoproterenol; (c) stimulation with 10^{-6} M carbamylcholine; (d) b plus c (similar amount of radioactivity) run together, to prove the identity of the phosphopeptides. Initial experiments in which the sample was introduced more to the middle of the plate had shown that no phosphopeptides with a pI-value of 3.5 or lower appeared which might be lost when applying the sample more to the corner of the plate.

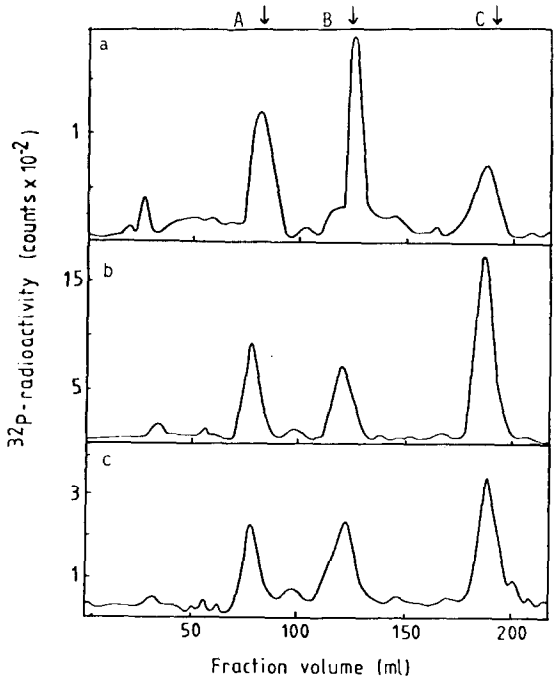


Fig. 3. Separation of tryptic phosphopeptides from unfractionated S6 by anion exchange chromatography. The S6 containing protein band (fig. 1) was excised from the gel, digested with trypsin and the phosphopeptides separated on DEAE–trisacrylate by a linear gradient from 50–400 mM NH_4HCO_3 (pH 8.6): (a) control state; (b) stimulation with 2×10^{-5} M isoproterenol; (c) stimulation with 10^{-6} M carbamylcholine.

were detected in the autoradiograph whereas after carbamylcholine treatment a third phosphorylated S6 spot appeared which was fainter. The absolute radioactivity in the S6 proteins analysed in the various experiments varied considerably so that a direct comparison of quantitative effects between isoproterenol and carbamylcholine stimulated phosphorylation of S6 cannot be made on the basis of the results presented in fig. 3,4. We have, however, observed that tri- and tetraphosphorylated species appeared more frequently after carbamylcholine stimulation than after stimulation by isoproterenol.

The number of phosphorylated serine residues per protein molecule was approximated from the anodic shift of the individual S6 derivatives (not shown, cf. [7]). The monophosphorylated S6 derivative showed two phosphopeptide peaks in the elution profile of the DEAE–Trisacryl column (fig. 4a,c,e). This result indicates that although each S6 molecule is monophosphorylated, two different serine residues have been modified. A similar elution profile was found by the analysis of monophosphorylated S6 protein from *Xenopus laevis* oocytes [21]. In the diphosphorylated S6 species from parotid glands an additional phosphopeptide eluted at higher ionic strength (fig. 4b,d,f). Thus all sites of the tryptic elution profile from total S6 protein were accounted for in these patterns of the individual S6 species. In the tri- and tetraphosphorylated S6 species, besides phosphopeptides B and C, several additional phosphopeptides appeared (fig. 4g), which may correspond to the additional spots depicted in fig. 2c.

Authors in [9] have analysed phosphopeptides from S6 obtained from reticulocytes with a method very similar to the one we have used. After phosphorylation of isolated reticulocyte ribosomal 40 S subunits in vitro with cAMPdPK, two phosphopeptides were found [9] which on the basis of their position correspond to our phosphopeptides A and C (fig.2). Labeling of S6 in intact reticulocytes in the presence of 8′-bromo-cAMP again led to an enhanced phosphorylation of only two phosphopeptides corresponding to our phosphopeptides A and C, whereas a third phosphopeptide corresponding in its position to our phosphopeptide B did not change its degree of phosphorylation in the presence of the nucleotide [9]. In our laboratory, corresponding results were obtained in preliminary experiments of in vitro phosphorylation of ribosomes from parotid glands by catalytic subunit of cAMPdPK. Extending these considerations to the findings in [10]. it seems well possible that our phosphopeptides A and C are the guineapig equivalents of the mono- and di-phospho derivatives of the rat S6 Arg–Leu–Ser–Ser–Leu–Arg sequence described by these authors.

On the other hand, in our system with intact cells an agonist involving cAMP as second messenger led to the formation of 3, and not only 2 phosphopeptides, and these 3 phosphopeptides were identical with those obtained after stimulation with an agonist involving calcium as second messenger. It therefore seems possible that S6 phosphorylation in the presence of isoproterenol is not, or not exclusively, catalysed by a cAMPdPK.

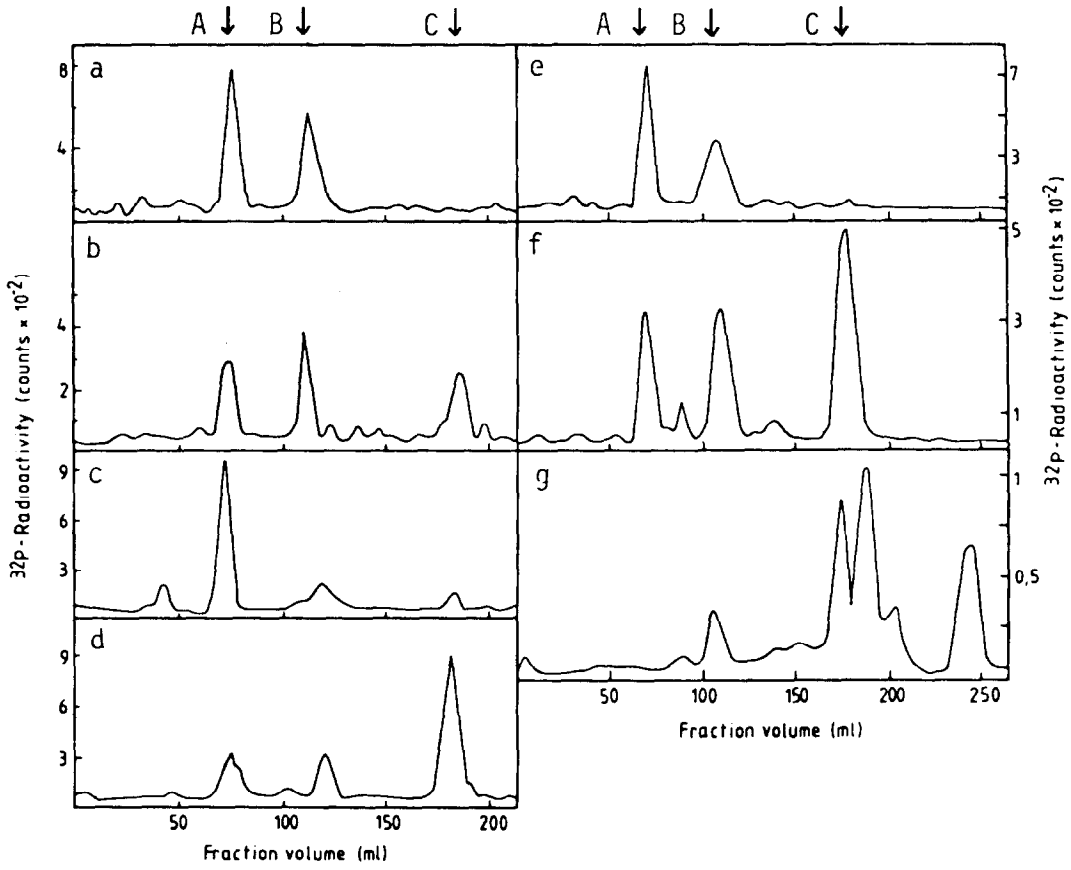


Fig. 4. Anion-exchange chromatography of phosphopeptides derived from S6 species containing increasing amounts of phosphate. The S6 species were resolved as in [7] by two-dimensional electrophoresis [18]. The protein spot from the individual derivatives from S6 were excised from the gel, digested with trypsin, and the phosphopeptides separated by DEAE–trisacrylate chromatography as in fig. 3. Phosphate incorporation into S6 was higher after carbamylcholine treatment compared to isoproterenol incubation in this particular experiment. The small peak in front of A in fig. 4c is an artifact due to the start of the gradient. (a,b) control state; (c,d) stimulation with 2×10^{-5} M isoproterenol; (e,f,g) stimulation with 10^{-6} M carbamylcholine; (a,c,e) mono-phosphorylated derivative; (b,d,f) di-phosphorylated derivative; (g) tri- and tetra-phosphorylated derivatives.

It should be stated also at this point that it cannot yet be excluded that the increased phosphorylation of the S6 protein following stimulation of secretion in exocrine organs results mainly or in part from a decreased activity of protein phosphatase(s) responsible for the dephosphorylation of the S6 protein. Both problems are currently under investigation.

The identical phosphopeptide pattern obtained in our system after stimulation with two different types of agonists contrasts with other systems where different agonists led also to different phosphopeptide patterns of S6, as shown for the effects of insulin *vs* dibutyryl-cAMP in HeLa cells [13] and for the effects of glucagon *vs* insulin in isolated hepatocytes [12].

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