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Identification of the site phosphorylated by casein kinase II in smooth muscle caldesmon

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Phosphorylation of avian gizzard caldesmon by casein kinase II was investigated. The enzyme incorporates about 1 mol of phosphate per mol of caldesmon. All sites of phosphorylation are located in short chymotryptic peptides with M_r 25–27 kDa or in the short N-terminal peptide formed after cleavage of chicken gizzard caldesmon at Cys^{153}. The primary structure of the tryptic peptide containing the main site of duck gizzard caldesmon phosphorylation is S-E-V-N-A-Q-N-X-V-A-E-D-E-T-K, where X is an unidentified residue, presumed to be phosphorserine. Thus, Ser^{73} is the main site phosphorylated by casein kinase II in avian gizzard caldesmon.

Caldesmon; Casein kinase II; Phosphorylation

1. INTRODUCTION

Caldesmon is an actin- and calmodulin-binding protein which seems to be involved in the regulation of contractile activity of smooth muscle and non-muscle motility [1–3]. Caldesmon is phosphorylated in vivo [4–6] and it is supposed that phosphorylation may affect the ability of caldesmon to regulate the actin and myosin interaction [7,8]. In vitro, caldesmon is phosphorylated by Ca-phospholipid-dependent protein kinase [9–11], Ca-calmodulin-dependent protein kinase [11,12] and by casein kinase II [11]. For understanding the physiological effect of phosphorylation it is desirable to locate the sites, which are phosphorylated by different protein kinases in caldesmon structure. Recently the sites phosphorylated in caldesmon by Ca-phospholipid-dependent- [13] and Ca-calmodulin-dependent protein kinases [14] were determined. The present paper is devoted to localization of the sites phosphorylated by casein kinase II in smooth muscle caldesmon.

2. MATERIALS AND METHODS

Caldesmon was isolated from duck or chicken gizzard according to Vorotnikov and Gusev [15]. Casein kinase of the second type was purified from rat liver by earlier described method [16]. Caldesmon was phosphorylated in the incubation mixture containing 8 mM Tris, 6 mM KH_2PO_4, 30 mM NaCl, 3 mM $MgCl_2$, 2 mM dithiothreitol and 100–200 μM ATP, containing [γ-^{32}P]ATP. The caldesmon concentration was equal to 0.4–0.6 mg/ml, whilst that of protein kinase did not exceed 30 μg/ml. Incubation for 1.5–2 h leads to incorporation of 0.4–0.6 mol of phosphate per mol (89 kDa) of caldesmon. The phosphorylated protein was subjected to chemical cleavage at Cys residues as described earlier [8,15]. The peptide mixture thus obtained was subjected to gel-electrophoresis in the presence of sodium dodecyl sulphate [17], and the peptides containing radioactive phosphate were determined by autoradiography [11,15].

For localization of the phosphorylation site, caldesmon, phosphorylated in the presence of [γ-^{32}P]ATP by casein kinase, was precipitated with 10% trichloroacetic acid. The precipitate was washed 3 times with 5% trichloroacetic acid and ether, and then dried. Caldesmon (1.4 mg) was suspended in 1.4 ml of a solution containing 50 mM NH_4HCO_3, 100 μM $CaCl_2$, and 1 mM dithiothreitol, and hydrolyzed by TPCK-treated trypsin (the ratio of caldesmon/trypsin was 50:1 w/w) for 4 h at 37°C. The resulting clear solution was subjected to HPLC performed on Waters system. For the initial separation the 4.6 × 250 mm Vydac 218TP54 C_{18} reverse-phase column was equilibrated with 0.1% aqueous solution of trifluoroacetic acid (TFA) as solvent A and peptides were eluted at a flow rate of 1 ml/min with linear gradients of solvents A and B (90% acetonitrile with 0.1% TFA). The gradients were as follows: 0% to 30 B over 60 min, followed by 30% to 50% B over 30 min, and finally 50% to 100% B over 10 min. Peptides were detected by monitoring the absorbance of the eluent at 215 and 280 nm. Individual peaks were collected manually and 25 μl aliquots were taken from each fraction for ^{32}P measurements using a Beckman LS 600 IC liquid scintillation counter.

Nine radioactive fractions, A to I (see Fig. 2), were concentrated and applied to HPLC on the same column for the second purification step. In this case 10 mM NH_4CH_3COO pH 6.5 was used as solvent A and the peptides were eluted with a linear gradient of solvent B (90% acetonitrile with 10 mM NH_4CH_3COO). For fraction A gradients were: 0% to 50% B over 45 min, and 50% to 100% B over 10 min. For fraction B, gradients were 0% to 40% B over 40 min, and 40% to 100% B over 15 min. Fraction B, the major phosphopeptide fraction, yielded a single well-resolved radioactive peak (Fig. 3) which was dried and prepared for sequencing. Fraction A yielded a partially purified radioactive peak, therefore it was subjected to additional purification on the 'narrow bore' 2.1 × 250 mm Vydac 218TP54 C_{18} column. The single well-resolved peak of fraction A was dried and prepared for sequenc-

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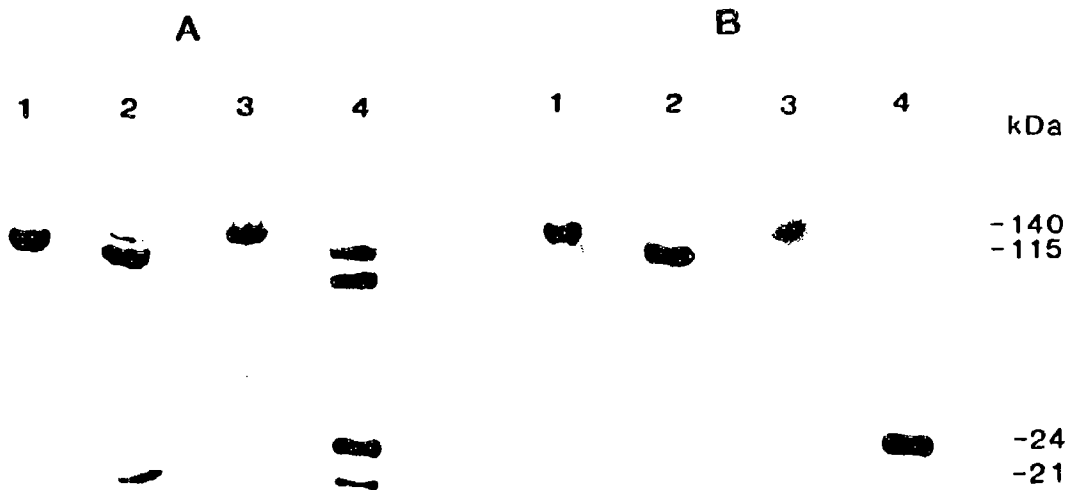

Fig. 1. (A) Electrophoretograms of native caldesmon phosphorylated by casein kinase II (1 and 3) and peptides obtained after chemical cleavage of phosphorylated caldesmon at cysteine residues (2 and 4). (B) Autoradiograms of the gels depicted in (A). Duck caldesmon, lanes 1 and 2, chicken caldesmon, lanes 3 and 4. The scale of apparent molecular masses are shown on the right.

ing. Fractions C through I yielded no well-defined radioactive peaks after their second HPLC chromatography, and were not analyzed in detail.

Peptide sequences were determined as described earlier [18] using an Applied Biosystems model 477A sequencer equipped with an on-line model 120A phenylthiohydantoin (PTH) analyzer. The PTH-derivatives of phosphorylated residues were not recovered but they could be identified by their absence. The PTH-derivatives of non-phosphorylated Ser and Thr were easily identified.

3. RESULTS

Prolonged incubation with casein kinase II leads to incorporation of about one mol of phosphate per mol of smooth muscle caldesmon [11]. The sites of phosphorylation are located in two short (M_r 25–27 kDa) peptides, which are liberated on initial stages of chymotryptic hydrolysis of caldesmon. These peptides are unable to interact with calmodulin and are therefore different from short C-terminal peptides of caldesmon. Taking into account these facts, we supposed that the sites which are phosphorylated by casein kinase II are located in short N-terminal fragments of caldesmon [11]. In order to verify this assumption, phosphorylated duck gizzard caldesmon was cleaved at Cys residues, and the peptides thus formed were analyzed by SDS-gel electrophoresis (Fig. 1). Under the conditions used, duck gizzard caldesmon formed two fragments with M_r of 115 and 21 kDa, only the former one being radioactive. Since the 21 kDa peptide is generated from the C-terminal part of caldesmon [15], we concluded that the sites

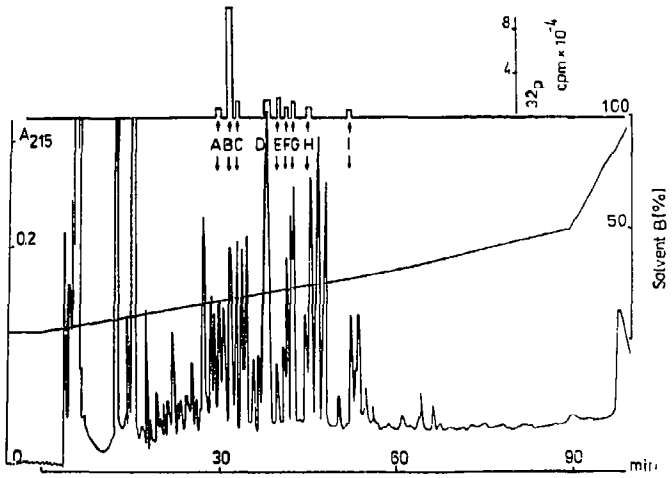

Fig. 2. Reverse-phase HPLC of tryptic peptides of duck gizzard caldesmon phosphorylated by casein kinase II. A to I denote the phosphopeptides in the order of their elution.

of casein kinase phosphorylation are located in the large N-terminal peptide of duck gizzard caldesmon. Chemical cleavage of chicken gizzard caldesmon at Cys residues lead to formation of 4 peptides with M_r of 110, 85, 24 and 22 kDa, of which the 110 and 24 kDa peptides were weakly and strongly radioactive. Chicken gizzard caldesmon has two Cys residues – Cys^{153} and Cys^{595} [19,20]. The short C-terminal peptide with M_r 22 kDa does not contain sites that are phosphorylated by casein kinase. At the same time, the short N-terminal peptide (residues 1–152) with M_r 24 kDa contains all the sites phosphorylated by casein kinase.

A detailed localization of the phosphorylation sites was performed after tryptic hydrolysis of phosphorylated caldesmon with concomitant purification of phosphorylated peptides by reverse-phase HPLC. Nine radioactive fractions, A to I, were detected when tryptic peptides of caldesmon were subjected to HPLC (Fig. 2). Fractions A and B contained 6 and 54% of the total radioactivity loaded on the column. Each of the other fractions (C through I) contained no more than 8% of the total radioactivity. These fractions yielded no well-defined radioactive peaks on the next step of purification and therefore could not be analyzed in detail. Fraction B (Figs. 2 and 3), the major phosphopeptide, yielded the sequence S-E-V-N-A-Q-N-X-V-A-E-D-E-T-K, where X is an unidentified amino acid presumed to be phosphorylated Ser (or Thr). The peptide eluted in fraction A possessed the same sequence plus an additional Arg at the C-terminus. Comparing these data with the primary structure of chicken gizzard caldesmon [19,20], we may conclude that the main site of phosphorylation is Ser^{73}.

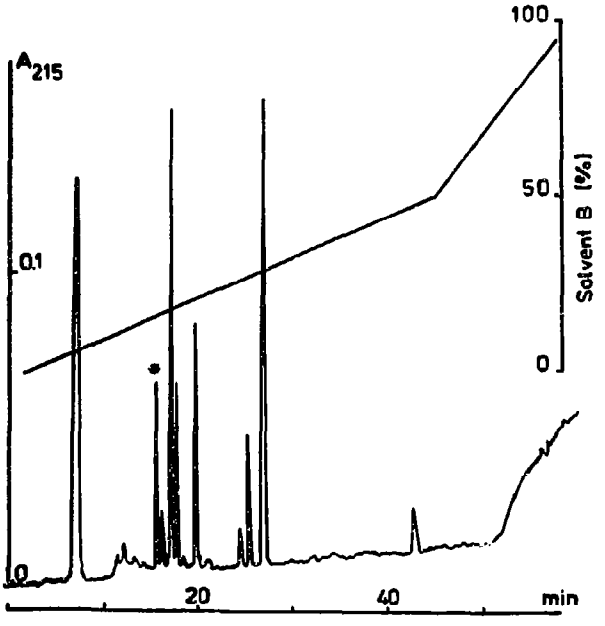

Fig. 3. Second reverse-phase HPLC of peptides eluted in fraction B of Fig. 2. The single radioactive peak is marked by the asterisk.

4. DISCUSSION

Casein kinase II is able to phosphorylate avian gizzard caldesmon. The sites of phosphorylation are located in the short N-terminal peptide (residues 1–153) of chicken gizzard caldesmon. Moreover, the sequence of the major phosphopeptide from duck gizzard caldesmon, S-E-V-N-A-Q-N-X-V-A-E-D-E-T-K-R, is similar to the corresponding tryptic peptide of chicken gizzard caldesmon which contains residues Ser^{66}–Arg^{81}. The only one difference between the two sequences is that Glu^{77} of chicken gizzard caldesmon is replaced by Asp in the duck gizzard protein. Sequencing of several tryptic peptides revealed that Ile^{139}, Val^{146} and Lys^{260} in chicken caldesmon were replaced by Thr, Ala and Arg respectively in duck gizzard caldesmon. Except for these conservative replacements, the N-terminal domain of chicken caldesmon contains Cys^{153}, whereas duck gizzard caldesmon lacks this residue in the corresponding position. C-terminal domains of different caldesmon species are also very similar. For example, a comparison of the primary structure of the 35 kDa peptides located in the C-terminal part of turkey and chicken gizzard caldesmon revealed only two conservative replacements out of 239 amino acid residues [18,21]. Thus, avian gizzard caldesmon seems to have rather a conservative primary structure.

It is known that casein kinase II predominantly phosphorylates Ser and Thr in the following sequence S/T-X-X-D/E (X is a neutral or negatively charged residue) [22,23]. These stretches are usually included in β-turns [22]. The sequence at Ser^{73} of duck gizzard caldesmon is S-V-A-E-D-E and the peptide containing residues 68–78 tends to form an unordered structure according to the predictions based on the method of Gibrat et al. [24]. Thus, Ser^{73} seems to be an ideal site for casein kinase II phosphorylation. It is worthwhile to mention that recently Ikebe and Reardon [14] found that Ca-calmodulin-dependent protein kinase phosphorylates 7 sites in smooth muscle caldesmon and Ser^{73} is phosphorylated at the highest rate. The consensus sequence at the sites phosphorylated by Ca-calmodulin-dependent protein kinase is as follows R-X-X-S [25], and differs from the corresponding sequence at Ser^{73} of caldesmon. This may indicate that the substrate specificity of Ca-calmodulin-dependent protein kinase is broader than expected earlier or that the specificity of this protein kinase is determined not only by the primary but also by the secondary or tertiary structure. One cannot completely exclude the possibility that the preparations of Ca-calmodulin-dependent protein kinase were contaminated by traces of casein kinase II, although according to Ikebe and Reardon [14] phosphorylation of Ser^{73} was completely dependent on the presence of Ca^{2+} and calmodulin.

There are several examples where casein kinase II is involved in cascade phosphorylation [23]. This means

that phosphorylation of certain sites catalyzed by casein kinase II may affect the phosphorylation of other sites, that are phosphorylated by protein kinase different from the former enzyme. This fact seems to be important, since the sites phosphorylated in caldesmon in vivo do not completely coincide with those phosphorylated in caldesmon in vitro [5,14].

Recently published data indicate that there are three sites – Ser^{73}, Ser^{602} and Ser^{741} – that are effectively phosphorylated by different protein kinases. Ca-calmodulin- and Ca-phospholipid-dependent protein kinases phosphorylate Ser^{602} and Ser^{741} [13,14]. Both these sites are located in the C-terminal part of caldesmon close to the sites involved in the interaction with calmodulin, actin and, probably phospholipids. At present there are controversial data on the effect of phosphorylation on the interaction of caldesmon with calmodulin, actin and phospholipids [10,15,26]. Ser^{73} is phosphorylated both by Ca-calmodulin-dependent protein kinase and casein kinase II. Sutherland and Walsh [8] reported that phosphorylation of caldesmon by Ca-calmodulin-dependent protein kinase leads to a decrease of the interaction between caldesmon and myosin. Taking into account that at least one site (Ser^{73}) phosphorylated by Ca-calmodulin-dependent enzyme overlaps with the site phosphorylated by casein kinase II, it is desirable to investigate the effect of phosphorylation of this site on the caldesmon interaction with myosin and other proteins of thin filaments. These studies are currently under way.

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