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Phosphorylation of smooth muscle caldesmon by three protein kinases: implication for domain mapping

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Phosphorylation of duck gizzard caldesmon by Ca^{2+}/phospholipid-dependent protein kinase, Ca^{2+}/calmodulin-dependent protein kinase and casein kinase II has been investigated. The Ca^{2+}/phospholipid-dependent protein kinase incorporates more than 3 mol phosphate per mol (140 kDa) caldesmon. All phosphorylation sites are localized in the actin- and calmodulin-binding peptide (40–45 kDa) supposed to be a part of the C-terminal domain of caldesmon. Casein kinase II phosphorylates only one site located in a short (25–27 kDa) peptide, presumably in the caldesmon N-terminal domain. The Ca^{2+}/calmodulin-dependent protein kinase phosphorylates two sites located in the N- and C-terminal domains of caldesmon.

Caldesmon; Calmodulin; Protein kinase; Phosphorylation; (Duck gizzard)

1. INTRODUCTION

Caldesmon is one of the major calmodulin- and actin-binding proteins, isolated from smooth muscle [1,2] and nonmuscular tissues [3,4]. Caldesmon is supposed to be involved in the Ca^{2+}-dependent regulation of actin and myosin interaction [5,6]. The Ca^{2+} phospholipid-dependent protein kinase (so-called protein kinase C) as well as the Ca^{2+} calmodulin-dependent protein kinase copurified with caldesmon are able to phosphorylate caldesmon [7,8]. Recent data indicate that phosphorylation of caldesmon may take place in vivo [4,6]; phosphorylation may affect the caldesmon-dependent regulation of actin and myosin interaction [8].

The aim of the present paper was to investigate the phosphorylation of caldesmon by a number of protein kinases and to locate the sites phosphorylated by these enzymes.

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2. MATERIALS AND METHODS

Duck gizzard caldesmon was isolated according to Lynch and Bretscher [2]. Calmodulin purified from bovine brain [9] was immobilized on CNBr-activated Sepharose. Rat brain protein kinase C was isolated by a slightly modified [10] method of Uchida and Filburn [11]. The partly purified Ca^{2+} calmodulin-dependent protein kinase from rat brain was obtained as described by McGuinness et al. [12], in which the stage of gel filtration was substituted by chromatography on phosphocellulose performed according to Karet and Schulman [13]. Casein kinases I and II were purified from rat liver cytosol [14]. Protein kinase activity was measured by incorporation of ^{32}P from [γ-^{32}P]ATP into histone H1 (for protein kinase C and Ca^{2+} calmodulin-dependent protein kinase) or casein (for casein kinases) according to Reimann et al. [15]. Caldesmon phosphorylation was performed in the following media: in the case of protein kinase C and Ca^{2+} calmodulin-dependent protein kinase – 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 mM $CaCl_2$ and either 120 μg/ml of a sonicated suspension of soybean phospholipids or 10–20 μg/ml of calmodulin; in the case of casein kinases – 8 mM Tris, 13 mM KH_2PO_4, 5 mM $MgCl_2$, and 2 mM dithiothreitol. The ATP concentration varied from 100 to 500 μM and that of enzymes from 10 to 30 μg/ml; caldesmon concentration was 0.4 mg/ml.

Phosphorylated caldesmon was digested by α-chymotrypsin (1–45 min, 30°C, protease/substrate weight ratio 1:1000) according to Szpacenko and Dabrowska [16]. The reaction was terminated by addition of phenylmethylsulfonyl fluoride to a

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final concentration of 2 mM. The peptide mixture was separated on gradient (7–20%) polyacrylamide gels in the presence of SDS [17]. For calculation of molecular mass, the following marker proteins were used: caldesmon (140 kDa), phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa). Autoradiography was performed on an RT-1 film varying the exposure time from 1 to 7 days. Protein concentration was determined either spectrophotometrically, taking $E_{276}^{1\%} = 5$ for caldesmon [18] and $E_{278}^{1\%} = 2$ for calmodulin [16], or by the method of Spector [19] standardized by human serum albumin.

3. RESULTS

Prolonged incubation of caldesmon with protein kinase C led to the incorporation of 3.0–3.5 mol phosphate per mol (140 kDa) protein (fig.1). These data fully agree with those of Umekawa and Hidaka [7], suggesting that the enzyme incorporates 7.7 mol phosphate per caldesmon dimer (300 kDa). For localization of phosphorylation sites, caldesmon was digested by chymotrypsin, and the peptides thus obtained were separated by SDS gel electrophoresis. Protein kinase C phosphorylated the sites located in both the large peptide (115–120 kDa) and a number of short peptides with 45, 40 and 19–21 kDa (fig.2). The kinetics of proteolysis indicate that the 19–21 kDa peptide may be the product of degradation of the 40 and 45 kDa peptides. Among other chymotryptic peptides only those of 40–45 and 19–21 kDa were not adsorbed on DEAE-cellulose under the conditions described by Fujii et al. [3]. The same peptides (as well as the 115–120 kDa peptide) were tightly adsorbed on immobilized calmodulin in the presence of Ca^{2+} and were eluted from the column with EGTA-containing buffers. The experimental results are consistent with published data, according to which the C-terminal fragment of caldesmon (40 kDa) can interact with actin and Ca^{2+}-saturated calmodulin [3,14,20]. Hence, protein kinase C phosphorylates three sites located in the short (40–45 kDa) C-terminal domain providing for caldesmon interaction with actin and calmodulin. This assumption is in agreement with the fact that calmodulin causes 40–60% inhibition of the Ca^{2+} phospholipid-dependent phosphorylation of caldesmon.

Casein kinase I did not phosphorylate caldesmon, whereas casein kinase II incorporated about 1 mol phosphate per mol protein (fig.1).

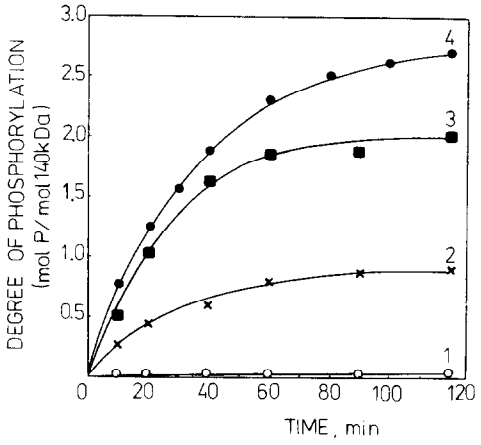

Fig.1. Phosphorylation of duck gizzard caldesmon by casein kinase I (1), casein kinase II (2), Ca^{2+} calmodulin-dependent protein kinase (3), and protein kinase C (4).

The sites phosphorylated by this enzyme are located in both large (90–120 kDa) and small (25–27 kDa) chymotryptic peptides of caldesmon (fig.2). Calmodulin does not affect caldesmon phosphorylation catalyzed by casein kinase II. The enzyme does not phosphorylate sites located in the 40–45 and 19–21 kDa peptides, i.e. in the C-terminal peptides of caldesmon. At the same time the 25–27 kDa peptides containing the sites phosphorylated by casein kinase II already appear during the earliest stages of proteolysis (fig.2). Thus, one may suppose that casein kinase II phosphorylates sites located in a rather short N-terminal peptide of caldesmon.

The Ca^{2+} calmodulin-dependent caldesmon kinase described by Ngai and Walsh [8], which is copurified with caldesmon, incorporates 2–4 mol phosphate per mol protein. A similar degree of phosphorylation was achieved when Ca^{2+} calmodulin-dependent protein kinase purified from rat brain was used (fig.1). The sites phosphorylated by this enzyme are located in peptides of 40–45 and 19–21 kDa, i.e. in the C-terminal peptides of caldesmon as well as in the 25–27 kDa peptides, i.e. presumably in the N-terminal peptides of caldesmon. Preliminary data published recently indicate that the sites phosphorylated by caldesmon-kinase and Ca^{2+} calmodulin-dependent protein kinase are similar or even identical [21]. A large excess of calmodulin

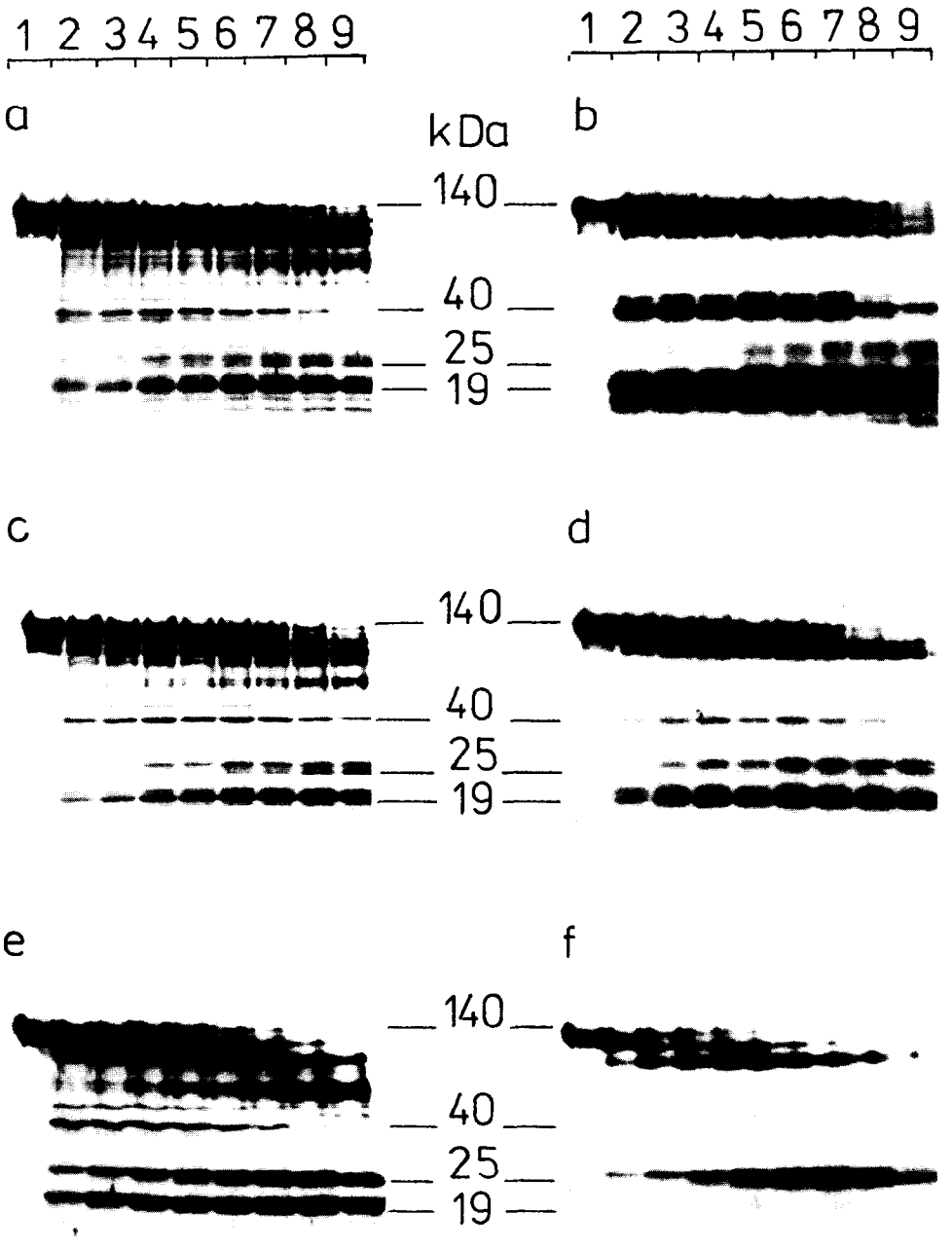

Fig.2. One-dimensional chymotryptic peptide maps of caldesmon phosphorylated by protein kinase C (a,b), Ca^{2+} calmodulin-dependent protein kinase (c,d), and casein kinase II (e,f). (a,c,e) Coomassie blue-stained gels; (b,d,f) autoradiograms. Lanes 1–9 represent electrophoretic patterns after 0, 1, 3, 5, 7, 10, 15, 30 and 45 min chymotrypsinolysis, respectively.

(calmodulin/caldesmon molar ratio, 10:1) inhibits phosphorylation of sites located in the C-terminal peptides (40–45 kDa) and does not influence phosphorylation of sites located in the N-terminal peptides (25–27 kDa) of caldesmon. This fact agrees with the assumption that some of the sites phosphorylated by the Ca^{2+} calmodulin-dependent protein kinase are located in the calmodulin-binding domain of caldesmon.

4. DISCUSSION

The present results suggest that three enzymes, i.e. protein kinase C, Ca^{2+} calmodulin-dependent protein kinase and casein kinase II, are able to phosphorylate caldesmon. One phosphorylation site is presumably located in the N-terminal fragment (25–27 kDa), whereas three are located in the C-terminal fragment (40–45 kDa) of caldesmon. The latter sites phosphorylated by protein kinase C and Ca^{2+} calmodulin-dependent protein kinase are located in the vicinity of the calmodulin-binding site of caldesmon. Therefore, under certain conditions, calmodulin can inhibit phosphorylation of these sites. Another group of sites phosphorylated by casein kinase II and Ca^{2+} calmodulin-dependent protein kinase is presumably located in the N-terminal fragment of caldesmon distant from the calmodulin-binding site; thus calmodulin does not interfere with the phosphorylation of these sites.

Localization of the sites of phosphorylation provides more detailed information on the domain organization of caldesmon discussed in the literature [3,16,20]. The data in fig.2 indicate that there are a few sites with an especially high susceptibility to chymotrypsinolysis. Hydrolysis at sites located in the C-terminal part of caldesmon leads to the formation of short peptides (19–21 and 40–45 kDa) containing the sites phosphorylated by protein kinase C. The N-terminal domain of caldesmon also contains a site susceptible to chymotryptic attack. Therefore, even during the early stages of proteolysis of caldesmon phosphorylated by casein kinase, the phosphorylated peptide of 25–27 kDa is liberated (fig.2). The central part of the caldesmon molecule is rather resistant to chymotrypsinolysis, and only long-term proteolysis causes degradation of the large central peptide (70–90 kDa) and the formation of short peptides, some of which may contain one of the sites phosphorylated by protein kinase C.

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