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Characterization of protein kinase C-mediated phosphorylation of the short cytoplasmic domain isoform of C-CAM

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Abstract C-CAM is a ubiquitously expressed cell adhesion molecule belonging to the carcinoembryonic antigen family. Two co-expressed isoforms, C-CAM-L and C-CAM-S, are known, having different cytoplasmic domains both of which can be phosphorylated in vivo. Here we have characterized the PKC-mediated phosphorylation of the short cytoplasmic domain isoform, C-CAM-S. Phorbol myristyl acetate induced phosphorylation of C-CAM-S in transfected CHO cells. Using synthetic peptides and Edman degradation we identified Ser^{449} as the PKC-phosphorylated amino acid residue. Binding experiments with modified peptides indicated that this phosphorylation decreases the ability of the cytoplasmic domain of C-CAM-S to bind calmodulin.

Key words: Calmodulin; Cell-cell adhesion molecule; Phosphorylation; PKC

1. Introduction

Members of the immunoglobulin superfamily [1] participate in cellular interactions and signaling processes necessary for the organization, structural integrity and functions of cells and tissues in multicellular organisms [2]. One of these molecules, C-CAM, is a transmembrane cell adhesion molecule, that is widely expressed in epithelia, vessel endothelia and hematopoietic cells [3,4]. C-CAM was first noted for its ability to mediate Ca^{2+}-independent cell adhesion in rat hepatocytes [5], where it appears as two major isoforms, C-CAM-L (C-CAM1) and C-CAM-S (C-CAM2) [3]. Due to differential splicing the cytoplasmic domain of C-CAM-L is larger (71–73 amino acids) than that of C-CAM-S (10–12 amino acids). While both C-CAM-L and C-CAM-S can mediate cell adhesion [6], several other functions have also been attributed to these molecules, especially to the larger isoform. C-CAM-L in rats has been described as a bile salt transporter, an ecto-ATPase, and a suppressor of tumor formation by prostate, bladder and breast carcinoma cells [3,7–9]. Also the mouse homologue of C-CAM-L, Bgp1, has been shown to suppress tumor growth of colon carcinoma cells in syngeneic mice [10]. Furthermore, murine C-CAM is the receptor for mouse hepatitis virus [11].

Cell adhesion molecules can be regulated in several different ways, which include both variations in expression levels, interactions with other membrane and cytoplasmic proteins, and covalent modifications [2,12]. C-CAM seems to be regulated by all of these mechanisms. During embryonic development the expression of C-CAM varies in a characteristic spatiotemporal pattern, and activation of different classes of leukocytes leads to altered surface expression patterns of C-CAM [3]. Protein tyrosine kinases and protein tyrosine phosphatases can bind to the cytoplasmic domain of C-CAM-L [3,13,14], suggesting important functions in signal induction and signal regulation. Of particular importance for the regulation of C-CAM activities is the finding that calmodulin can bind to the cytoplasmic domains of both C-CAM-L and C-CAM-S in a calcium-regulated manner, resulting in altered C-CAM self-association and dimerization [15,16]. Another feature important for regulation of C-CAM is that both C-CAM-L and C-CAM-S can be phosphorylated in vivo [13,17–20]. C-CAM-L, that has a larger cytoplasmic domain with more potential phosphorylation sites, has been shown to be phosphorylated to a greater extent than C-CAM-S.

Most attention has been focused on the cytoplasmic domain of C-CAM-L, which can be phosphorylated in vivo by both protein kinase C (PKC) and tyrosine kinases [13,18,19] on Ser^{503} and Tyr^{488}, respectively. The effects of phosphorylation on the activities of C-CAM-L are not well understood, but phosphorylation of Ser^{503} and Tyr^{488} appears to participate in the regulation of transmembrane bile salt transport [18]. Tyrosine phosphorylation of C-CAM-L does not, however, seem to affect its cell adhesion activity [20]. Less is known about the phosphorylation of C-CAM-S, that lacks phosphorylatable tyrosine residues. Therefore we investigated the serine/threonine phosphorylation pattern of this isoform. We report that C-CAM-S can be phosphorylated on a specific membrane-proximal serine residue by PKC, and present data indicating that PKC-mediated phosphorylation of this isoform alters its potential to bind calmodulin.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized on cellulose membranes using the SPOT-system (Cambridge Research Biochemical, Northwich, England) [21,22] as previously described [15]. For peptides that were to be sequenced, the acetylation step was omitted.

2.2. Phosphorylation of synthetic peptides

Phosphorylation of synthetic peptides was performed with protein kinase C (PKC) as previously described [23]. PKC-β was prepared from pig spleen, as described by Ferrari et al. [24]. The kinase specific activity was 300 U/mg; one unit was defined as the amount of enzyme that transferred 1 nmol of phosphate from ATP to histone (H1) in one minute (1 mg histone H1/ml incubation buffer).

2.3. Amino acid sequence determination

Manual Edman degradation of the phosphorylated membrane-bound peptides was performed as described by Sullivan and Wong [25], with a few modifications. Before sequencing, the peptide-contain-

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Abbreviations: C-CAM, cell-cell adhesion molecule; PMA, phorbol myristyl acetate; PKC, protein kinase C

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ing membranes were incubated in trifluoroacetic acid (TFA) at 50°C for 10 min and were then washed with methanol and dried. For each degradation cycle each membrane-spot was incubated at 50°C for 10 min, in 0.5 ml of coupling solution (methanol:water:triethylamine: phenylisothiocyanate; 7:1:1:1, v/v). The coupling solution was removed, and the membranes were washed five times in methanol and dried. The membranes were incubated at 50°C for 5 min, in 0.5 ml of TFA, and then again at room temperature for 1 min, in 1 ml of TFA: 42.5% H_3PO_4 (9:1, v/v). Finally, the membranes were washed with methanol, dried and subjected to a new degradation cycle. The two TFA wash solutions from each degradation cycle were combined and analyzed for ^{32}P-activity. Also the remaining peptide-bound ^{32}P-activity was measured after each degradation cycle. ^{32}P-activity was determined as Cerenkov radiation.

2.4. Calmodulin binding to synthetic peptides

Binding of ^{125}I-labeled calmodulin to membrane-bound synthetic peptides was determined as previously described [15].

2.5. Phosphorylation of cellular C-CAM

C-CAM-S-transfected CHO Pro5 cells (American Tissue Culture Collection) [6] were seeded in 60 mm tissue culture dishes (1×10^6 cells per dish) on day 1, and cultured overnight. On day 2 the cells (2×10^6 cells per dish) were washed in phosphate-free MEM medium (Gibco BRL), and incubated in 2 ml phosphate-free MEM, 8% fetal calf serum and 1 mCi [^{32}P]orthophosphate (NEN) for 4 h at 37°C. Phorbol myristyl acetate (PMA) was added to some dishes to a final concentration of 20 nM, and the cells were incubated for another 15 min. The medium was then removed, the cells were washed with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, and lysed for 60 min at 4°C in 0.5 ml of 1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 2 mM sodium fluoride, 2 mM sodium pyrophosphate, 2 mg/ml aprotinin, 5 mg/ml leupeptin, 0.4 mg/ml pepstatin, 200 mg/ml (4-amidinophenyl)-methanesulfonylfluoride (APMSF), 10 mM Tris-HCl, pH 7.4. The lysate was centrifuged in an Eppendorf centrifuge, and the supernatant was precipitated with anti-C-CAM antiserum (anti-C-CAM10 in [4]) or preimmune immunoglobulins, after pretreatment with preimmune serum. The immunoprecipitates were then analyzed by SDS-PAGE, followed by both autoradiography and immunoblotting with anti-C-CAM antibodies and alkaline phosphatase-conjugated secondary antibodies.

3. Results and discussion

The phosphorylation sites in C-CAM-S have not, until now, been identified. In all C-CAM expressing cells analyzed so far, C-CAM-L and C-CAM-S are co-expressed [26], a fact which complicates a detailed biochemical analysis, as the two isoforms are difficult to separate. In this investigation we therefore analyzed transfected CHO cells expressing only the C-CAM-S isoform. Since the cytoplasmic domains of both C-CAM-S and C-CAM-L contain putative phosphorylation sites for PKC (Fig. 1) [27] we investigated if PKC can phosphorylate C-CAM-S in vivo by incubating the cells with radioactive orthophosphate and the PKC-stimulatory drug PMA. The cells were lysed and the lysates were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. No ^{32}P-labeled proteins were specifically immunoprecipitated in C-CAM-S-transfected, unstimulated cells (Fig. 2, lanes A and C), but C-CAM-S became strongly phosphorylated in the PMA-stimulated cells (Fig. 2, lane B). Control immunoglobulins did not recognize any phosphorylated proteins in PMA-stimulated cells (Fig. 2, lane D). Thus, activated PKC seems to phosphorylate the cytoplasmic domain of C-CAM-S in vivo.

In order to identify the amino acids that were phosphorylated by PKC, we used purified PKC-β for phosphorylation of synthetic decapeptides corresponding to different regions of the cytoplasmic domains of C-CAM. The peptides were synthesized on cellulose membranes; such peptides have recently been reported to be good substrates for protein kinases with-

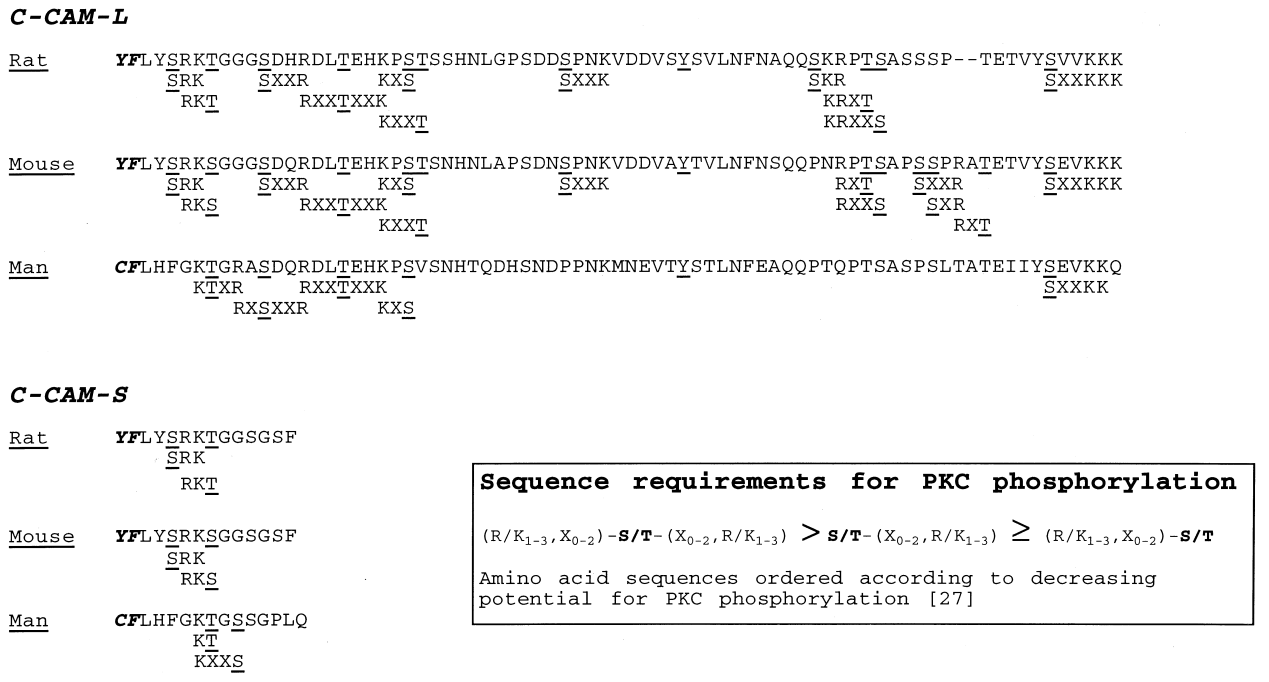

Fig. 1. Amino acid sequences and potential phosphorylation sites of C-CAM-L and C-CAM-S cytoplasmic domains. The amino acid sequences of the cytoplasmic domains of C-CAM-L and C-CAM-S of rat, mouse and human origin, are shown in single letter code. The two most N-terminal amino acids (shown to the left in bold and italic font) are believed to be members of the transmembrane region. The most N-terminal serine residue shown in the rat and mouse sequences is S^{449}. Sequence requirements for potential PKC phosphorylation sites, according to Kennelly and Krebs [27], are indicated in the inset. Potential PKC-phosphorylatable serine and threonine residues in the C-CAM sequences are underlined. The C-CAM-L tyrosine residue (Y^{488}), shown to be phosphorylated in vivo, is also underlined.

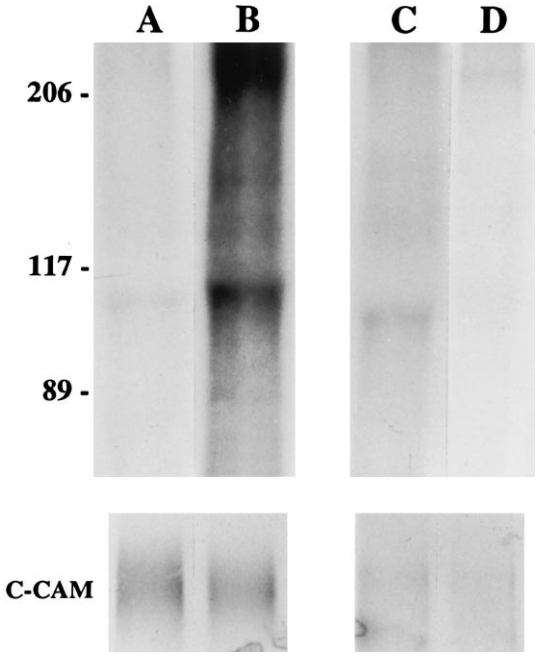

Fig. 2. PMA-stimulated phosphorylation of C-CAM-S in CHO cells. Triton X-100 extracts from C-CAM-S-transfected CHO cells, incubated with [^{32}P]orthophosphate, were precipitated with anti-C-CAM antibodies (A and B) or preimmune immunoglobulins (C and D). The precipitated samples were analyzed by SDS-PAGE and autoradiography. The migration positions of molecular weight markers are indicated: Untreated cells (A and C); PMA-treated cells (B and D). The samples were also immunoblotted with anti-C-CAM antibodies (shown below each lane).

out steric hindrance of enzyme access to the most C-terminal amino acids [23,28]. The phosphorylated peptides were subjected to manual Edman degradation and the amounts of released and remaining [^{32}P]phosphate were determined after each degradation cycle (Fig. 3A). Analysis of two different decapeptides, staggered by two amino acids, revealed that PKC primarily phosphorylated the serine residue at position 449 in peptides corresponding to the C-CAM-S sequence (Fig. 3B). PKC requires positively charged arginine and/or lysine residues in the vicinity of the phosphorylatable serine or threonine residues for optimal activity (Fig. 1) [27]. In order to analyze the specificity of the PKC-catalyzed phosphorylation we therefore synthesized peptides in which the arginine and lysine residues (Arg^{450} and Lys^{451}) in the C-CAM-S cytoplasmic domain were replaced with alanine residues. These peptides showed drastically lower phosphorylation levels (data not shown), demonstrating that the phosphorylation of Ser^{449} is in agreement with the known specificity of PKC. Because the membrane-proximal region of the cytoplasmic domain of C-CAM-L is similar in sequence to that of C-CAM-S (Fig. 1), we also phosphorylated synthetic peptides with C-CAM-L sequences and found that the corresponding serine residue (Ser^{449}) in C-CAM-L could also be specifically phosphorylated by PKC (data not shown).

The specific phosphorylation of one serine residue in the cytoplasmic domain of C-CAM-S by PKC suggests that PKC-mediated phosphorylation may be important for regulation of C-CAM-S function. While the cytoplasmic domain of C-CAM-L can interact with several signaling molecules, the cytoplasmic domain of C-CAM-S has so far only been found to be able to bind calmodulin [15]; in fact, calmodulin binding is the only known activity of the C-CAM-S cytoplasmic domain. Therefore, we thought it to be of interest to investigate if phosphorylation of C-CAM-S interferes with binding of calmodulin.

We first measured calmodulin binding to synthetic peptides that were phosphorylated in vitro by PKC. On average, PKC phosphorylation decreased calmodulin binding to the peptides, but the variation in the calmodulin binding levels between experiments was too large to allow precise analysis of these experiments. This variation was probably due to adsorption of components in the PKC preparation to the peptide-containing cellulose membranes, with subsequent influence on calmodulin binding to the membranes. We therefore took another approach, and synthesized peptides in which phosphorylation was simulated by replacing the serine and threonine residues with negatively charged glutamic acid residues. Calmodulin binding decreased to 25% and 16%, respectively, following these substitutions at S^{449} in C-CAM-L and C-CAM-S peptides (Table 1). When two or more serine/threonine residues were replaced by glutamic acid residues, calmodulin binding was completely abolished. To verify that this decrease in binding level was caused by the introduced negatively charged residues and not by structural changes in the peptide or the loss of a hydroxyl group at the position of the serine/threonine residues, a large number of peptides were synthesized, in which each amino acid residue systematically was changed to an alanine residue. Strikingly, only when one or both of the two basic residues (Arg^{450} and Lys^{451}) were substituted, there was a significant change in calmodulin binding (data not shown). Both the arginine and the lysine residues are required for calmodulin binding [15]. Exchange of the serine/threonine residues for alanine did not alter the level of calmodulin binding. This is thus compatible with the idea that phosphorylation of C-CAM at a specific serine residue can abolish calmodulin binding by the introduction of negative charges.

As C-CAM-S has been demonstrated to mediate cell-to-cell adhesion of the transfected CHO cells it was of interest to see if PKC-catalyzed phosphorylation would affect its adhesive function. We therefore investigated how PMA affected the aggregation of untransfected and C-CAM-S-transfected CHO cells, and found that PMA stimulated aggregation to

Table 1
Binding of calmodulin to C-CAM peptides

	Peptide sequence	% Binding
C-CAM-L	LYSRKTGGGS	100
	LY**E**RKTGGGS	25
	LYSRK**E**GGGS	25
	LYSRKTGGG**E**	51
	LY**E**RK**E**GGG**E**	2
C-CAM-S	SRKTGGSGSF	100
	ERKTGGSGSF	16
	SRK**E**GGSGSF	7
	SRKTGG**E**GSF	13
	SRKTGGSG**E**F	24
	ERK**E**GG**E**G**E**F	2

Serine and threonine residues were replaced by glutamic acid in synthetic C-CAM peptides, and binding of calmodulin to the peptides was measured. The substituted glutamic acid residues are indicated by a bold 'E'. The level of calmodulin binding to each modified peptide is given as a percentage of binding to the corresponding unmodified peptide.

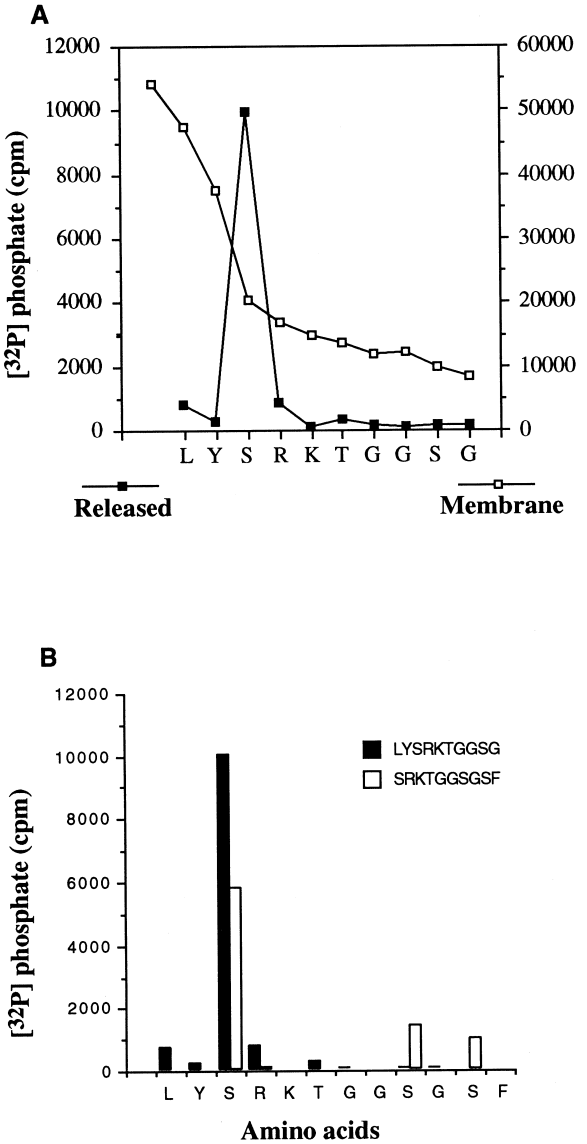

Fig. 3. Sequencing of phosphorylated synthetic C-CAM peptides. Decapeptides were phosphorylated by PKC-β with [32P]ATP and subjected to manual Edman degradation. The released and remaining, membrane-bound [32P]phosphate was measured after each degradation cycle. A: Released (filled boxes, left ordinate) and remaining (open boxes, right ordinate) [32P]phosphate, from a single decapeptide, corresponding to the cytoplasmic domain of C-CAM-S. B: Released [32P]phosphate from two different C-CAM-S decapeptides, staggered by two amino acid residues.

a large extent (data not shown). However, both the untransfected and the transfected cells were stimulated to the same extent, and detailed analysis using adhesion-blocking antibodies against C-CAM revealed that PMA did not affect the C-CAM-S-mediated adhesion. Since calmodulin can bind to the cytoplasmic domains of C-CAM when the calcium concentration is increased we also investigated if increasing the intracellular calcium concentration by addition of calcium ionophores would affect adhesion. A slight inhibitory effect was observed, but also in this case it could not be clearly distinguished from the effects on untransfected cells. Therefore, we conclude that neither phosphorylation nor calmodulin binding influences the intercellular adhesive activity of C-CAM-S in transfected CHO cells.

Since PKC-mediated phosphorylation of C-CAM-S does not appear to influence its basic adhesive activity, phosphorylation of C-CAM-S probably has other regulatory effects. One possibility is that it might influence interactions with other molecules, as demonstrated for calmodulin. Phosphorylation might also affect the supramolecular organization of C-CAM. It has been demonstrated that both C-CAM-L and C-CAM-S can form homodimers in the plane of the membrane in epithelial cells [16]. C-CAM dimer formation can be regulated by the cells, and the extent of dimers varies between different cell types. Calmodulin seems to be one factor that can regulate C-CAM dimers, as binding of calmodulin leads to dimer dissociation [16]. Accordingly, phosphorylation of the cytoplasmic domain of C-CAM-S might be important for regulating C-CAM dimerization, both by interfering with calmodulin binding and perhaps by directly influencing the affinity between C-CAM monomers. Regulation of C-CAM dimerization might have important effects on the signal regulatory functions of C-CAM [3].

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