Identification of the Serine Residue Phosphorylated by Protein Kinase C in Vertebrate Nonmuscle Myosin Heavy Chains†

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ABSTRACT: Two-dimensional mapping of the tryptic phosphopeptides generated following in vitro protein kinase C phosphorylation of the myosin heavy chain isolated from human platelets and chicken intestinal epithelial cells shows a single radioactive peptide. These peptides were found to comigrate, suggesting that they were identical, and amino acid sequence analysis of the human platelet tryptic peptide yielded the sequence -Glu-Val-Ser-Ser(PO_4)-Leu-Lys-. Inspection of the amino acid sequence for the chicken intestinal epithelial cell myosin heavy chain (196 kDa) derived from cDNA cloning showed that this peptide was identical with a tryptic peptide present near the carboxyl terminal of the predicted α-helix of the myosin rod. Although other vertebrate nonmuscle myosin heavy chains retain neighboring amino acid sequences as well as the serine residue phosphorylated by protein kinase C, this residue is notably absent in all vertebrate smooth muscle myosin heavy chains (both 204 and 200 kDa) sequenced to date.

Protein kinase C is a ubiquitous calcium-dependent kinase that is thought to play a major role in signal transduction in a variety of cells (Nishizuka, 1989). Among the numerous substrates that have been shown to be phosphorylated by protein kinase C both in vitro and in situ are the heavy and light chains of vertebrate nonmuscle myosin. The most common form of vertebrate nonmuscle myosin is a hexamer composed of a pair of heavy chains (196 kDa) and two pairs of light chains (20 and 17 kDa). Previous work has demonstrated that the 20-kDa light chain of nonmuscle myosin (Kawamoto et al., 1989; Ludowyke et al., 1989; Ikebe & Reardon, 1990; Carroll & Wagner, 1989) as well as smooth muscle myosin isolated from a variety of sources can be phosphorylated in vitro by protein kinase C (Nishikawa et al., 1984; Umemoto et al., 1989). The sites of phosphorylation have been identified in the smooth muscle light chains as serine-1, serine-2, and threonine-9 (Bengur et al., 1987; Ikebe et al., 1987). These sites appear to be conserved between the smooth muscle and nonmuscle 20-kDa myosin light chains (Kawamoto et al., 1989; Kumar et al., 1989). In situ phosphorylation of the serine sites on the light chains has been reported following treatment of smooth muscle fibers (Kamm et al., 1989; Singer et al., 1989; Sutton & Haeberle, 1990) and intact platelets (Kawamoto et al., 1989; Naka et al. 1983) with phorbol ester. The physiologic significance of these phosphorylations, if any, is unknown, although in vitro experiments have demonstrated that phosphorylation of the 20-kDa myosin light chain by protein kinase C decreases the affinity of myosin for myosin light chain kinase (Nishikawa et al., 1984).

In addition to phosphorylating the 20-kDa light chain of myosin in vertebrate nonmuscle and smooth muscle cells, protein kinase C has also been shown to phosphorylate the 196-kDa myosin heavy chain (MHC)[1] both in situ and in vitro. In situ phosphorylation of the MHC has been demonstrated in rat basophil leukemic cells (RBL-2H3 cells) following IgE receptor aggregation (Ludowyke et al., 1989). The stoichiometry of phosphorylation was 1.0 mol of phosphate/mol of MHC. The heavy chain of human platelet myosin can be phosphorylated by protein kinase C, both in vitro and following treatment of human platelets with phorbol ester (Kawamoto et al., 1989). The single tryptic phosphopeptides generated following in situ and in vitro phosphorylation of human platelet MHC were found to comigrate by using two-dimensional peptide mapping (Kawamoto et al., 1989), and these peptides also comigrated with the single tryptic phosphopeptide generated from RBL-2H3 myosin that was phosphorylated in situ (Ludowyke et al., 1989).

The presence of an apparent single serine residue that could be stoichiometrically phosphorylated by protein kinase C in vertebrate nonmuscle MHCs prompted us to investigate its exact location in the MHC. We were aided by the availability of the entire amino acid sequence for a nonmuscle MHC from chicken intestinal epithelial cells, derived by cDNA cloning (Shohet et al., 1989), as well as by a partial amino acid sequence for a human nonmuscle MHC, also derived by cDNA cloning (Saez et al., 1990). Our results show that vertebrate nonmuscle myosins contain a single serine residue that can be phosphorylated by protein kinase C near the carboxyl-terminal end of the predicted α-helix of the myosin rod. Although this site appears to be conserved in vertebrate nonmuscle MHCs

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[1] Abbreviations: MHC, myosin heavy chain; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; HPLC, high-pressure liquid chromatography; IDA, iminodiacetic acid.

from a variety of species, it is not present in the vertebrate smooth muscle MHC and, thus, constitutes a significant difference between vertebrate smooth muscle and nonmuscle MHCs. A preliminary report on this work has been made.[2]

Materials and Methods

Preparation of Proteins. Myosin was purified from fresh human platelets (Sellers et al., 1988). Chicken intestinal epithelial cell myosin was a gift of Kathy Collins (Whitehead Institute, MIT, Cambridge, MA). Rat brain protein kinase C was prepared as described (Huang et al., 1986) and, in some cases, was a gift of K. P. Huang (NICHHD, Bethesda, MD).

Phosphorylation and Tryptic Digestion. The extent of incorporation of ^{32}P from [γ-^{32}P]ATP into MHCs and light chains was monitored by SDS–PAGE. Appropriate slices were excised from 12.5% polyacrylamide gels, and ^{32}P was quantitated by liquid scintillation counting. Human platelet myosin (7.5 mg total, 1.5 mg/mL) or chicken intestinal epithelial cell myosin (1.35 mg/mL) was incubated with rat brain protein kinase C for 1 h at 30 °C in 40 mM Tris-HCl (pH 7.5), 0.24 mM $CaCl_2$ (in excess over EGTA), 1.6 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (0.2 mCi/mL), 24 mM NaCl, 0.6 mM DTT, 80 μg/mL phosphatidylserine, and 10 μg/mL diacylglycerol. The reaction mixture was concentrated by the addition of ammonium sulfate (60% saturation), solubilized, and dialyzed 2 h against 40 mM NaCl, 10 mM MOPS (pH 7.5), 1 mM EGTA, 1 mM EDTA, 50 mM NaF, 50 mM sodium pyrophosphate, and 1 mM DTT.

The MHCs were separated from the light chains and from protein kinase C by SDS–PAGE. The briefly stained band of MHC was excised from the 5% polyacrylamide preparative gel (120 mm × 140 mm × 1.5 mm) and washed three times in 25% 2-propanol (10 mL each) followed by three washes in 10% methanol (10 mL each). The lyophilized slice was crushed and suspended in 8 mL of 50 mM NH_4HCO_3 (pH 8.3). TPCK–trypsin was added in aliquots over 48 h at 37 °C to a final concentration of 2 mg/mL. The solution was aspirated and the gel rinsed in 50 mM NH_4HCO_3. The combined solutions were lyophilized, resuspended in H_2O, and dried down a total of three times. Digestion proceeded until no radioactivity could be detected in the crushed gel slices.

Peptide Analysis and Purification. Aliquots of the MHC tryptic digest were analyzed by two-dimensional thin-layer electrophoresis and chromatography (Ludowyke et al., 1989). The phosphopeptide was separated preparatively from the tryptic peptide mixture by chromatography on Fe^{3+}-IDA–Sepharose (Muszynska et al., 1986). The tryptic digest of MHC was suspended in 4 mL of 0.1 M acetic acid and applied to a 3-mL column of Fe^{3+}-IDA–Sepharose. The column was eluted with successive steps of 0.1 M acetic acid, 0.1 M sodium acetate, pH 5.0, and 1% ammonium acetate, pH 6.3. The phosphopeptide was eluted with 1% ammonium acetate, pH 8.3. Fractions of 0.5 mL were collected, and A_{280} was measured. ^{32}P was determined by liquid scintillation counting. The pooled radioactive peak was lyophilized, suspended in H_2O, and redried.

Sequence Analysis. Amino acid analysis of the isolated phosphopeptide fractions was carried out by using a Hewlett-Packard Amino-Quant system after hydrolysis in aliquots by gaseous HCl. Sequences were determined by using an Applied Biosystems 470A instrument equipped with an on-line 120A HPLC. Conversion of phosphoserine to *S*-ethylcysteine was carried out as described by Meyer et al. (1986).

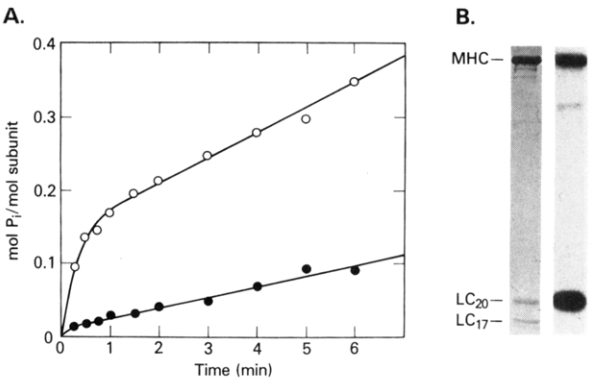

FIGURE 1: Initial rates of in vitro phosphorylation of human platelet myosin by protein kinase C. (A) Platelet myosin was incubated with protein kinase C as described under Materials and Methods. At the indicated times, the reaction was stopped by boiling an aliquot in SDS–polyacrylamide gel sample buffer (Laemmli, 1970), following which the sample was electrophoresed in 12.5% polyacrylamide–SDS gels. The bands corresponding to the MHC and 20-kDa light chain were excised, and ^{32}P radioactivity was assayed in a liquid scintillation counter. *Open circles* indicate phosphate incorporation into the 20-kDa myosin light chain, and *closed circles* indicate incorporation into the MHC. (B) 12.5% polyacrylamide–SDS gel stained with Coomassie blue (left) and autoradiogram of the myosin phosphorylated by protein kinase C (right). MHC, myosin heavy chain; LC_{20} and LC_{17}, 20- and 17-kDa myosin light chains.

Results

Human platelet myosin and chicken intestinal epithelial cell myosin were phosphorylated in vitro by incubation with protein kinase C. Figure 1A is a time course showing the initial rates of phosphorylation of the platelet MHC and 20-kDa light chain. In vitro, under the conditions used, the light chains are phosphorylated 2.5 times faster than the heavy chains. If the reaction is allowed to continue, the light chains can be phosphorylated up to 1.4 mol of phosphate/mol of light chain and the heavy chains up to 0.6 mol of phosphate/mol of MHC. Figure 1B shows a Coomassie blue stained gel and autoradiogram of platelet myosin phosphorylated by protein kinase C. Two-dimensional tryptic phosphopeptide maps of the MHC show that a single tryptic peptide is phosphorylated in both human platelet and chicken intestinal epithelial cell myosin (Figure 2). As shown, when the tryptic digests are analyzed on the same thin-layer plate, the tryptic phosphopeptides comigrate.

The tryptic peptide digest of phosphorylated platelet MHC was chromatographed on Fe^{3+}-IDA–Sepharose (Figure 3). Almost all of the tryptic peptides elute from the column without binding during the initial wash with the loading buffer. There is no radioactivity detected in this initial protein peak. A single peak of radioactivity containing 92% of the applied radioactivity was eluted from the column at pH 8.3. The peak of radioactivity was pooled and isolated by HPLC on a C_{18} μBondapak column (data not shown).

The peptide was sequenced, and the phosphoserine was localized as follows. The radioactive peak was quantified by amino acid analysis, and an amount estimated to be 50 pmol was sequenced on an Applied Biosystems 470A sequencer equipped with a 120A on-line HPLC. The sequence obtained (see Table I) was Glu-Val-Ser-Ser-Leu-Lys. In this instrument the PO_4 remains bound to the filter in the sample cartridge, and therefore we could not tell whether Ser-3 or Ser-4, or both, was phosphorylated. We then converted the PO_4-Ser to *S*-ethylcysteine by β-elimination and treatment with ethanethiol (Meyer et al., 1986) and repeated the sequencing. As shown

[2] Seventh International Conference on Cyclic Nucleotides, Calcium and Protein Phosphorylation, Kobe, Japan, Oct 1989.

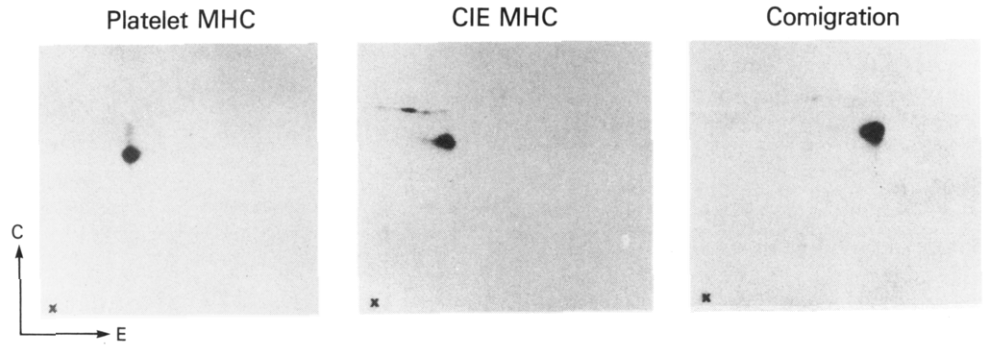

FIGURE 2: Peptide mapping of human platelet and chicken intestinal epithelial cell MHC following phosphorylation by protein kinase C. The radioactively labeled MHCs were excised from SDS–polyacrylamide gels, digested with trypsin as outlined under Materials and Methods, and analyzed by thin-layer electrophoresis (E) and ascending chromatography (C) as described in Ludowyke et al. (1989). Phosphopeptides from the platelet MHC (platelet MHC) and chicken intestinal epithelial cell MHC (CIE MHC) were detected by autoradiography. The rightmost panel (comigration) is a 1:1 mixture (cpm) of the phosphopeptides from both MHCs.

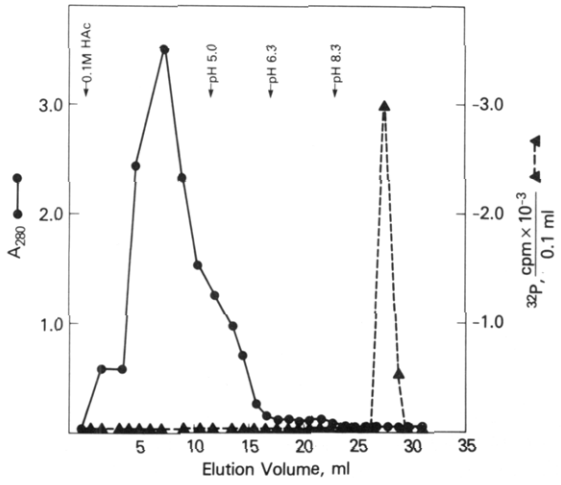

FIGURE 3: Separation of the platelet MHC tryptic peptides on Fe^{3+}-IDA–Sepharose. Four milliliters of the tryptic digest in 0.1 M acetic acid was applied to a 3-mL column of Fe^{3+}-IDA–Sepharose equilibrated with 0.1 M acetic acid. Elution of the column with buffers of varying pH was as shown on the diagram (see Materials and Methods for buffer composition). *Closed circles* indicate absorption at 280 nm; *closed triangles* indicate radioactivity as assayed by liquid scintillation counting.

Table I: Sequence Analysis of the Phosphopeptide[a]

	before conversion		after conversion	
step	Pth-aa	pmol	Pth-aa	pmol
1	Glu	30	Glu	34
2	Val	18	Val	25
3	ΔSer	5.5[b]	ΔSer[c]	4.3
			S-Et-Cys	0
4	ΔSer	6.3[b]	ΔSer[c]	3.2
			S-Et-Cys	3.2
5	Leu	10	Leu	6
6	Lys	4	Lys	3

[a] Approximately 50 pmol was applied for each determination. One determination was carried out on the peptide as isolated and one after treatment under conditions that led to conversion of PO_4-Ser to *S*-ethyl-Cys. [b] At steps 3 and 4, both Pth-Ser and Pth-ΔSer (the DTT adduct of dehydroalanine) were seen. We do not have standards for ΔSer, and values shown were obtained by using the value for Pth-His which elutes near Pth-ΔSer. ΔSer is generated from both Ser and PO_4-Ser. [c] After conversion, step 3 looked very much like step 3 before conversion, while step 4 contained *S*-Et-Cys. Pth-*S*-Et-Cys emerges just before diphenylthiourea in our HPLC system. The value shown was estimated by using the standard for Pth-Met, a homologue of *S*-Et-Cys.

in Table I, *S*-ethylcysteine was seen at step 4 and not at step 3. The low yield suggests that the reaction is not quantitative, consistent with other reports (Murakami et al., 1990), but the assignment of PO_4-Ser to step 4 seems definitive.

The amino acid sequence of the human platelet MHC tryptic peptide determined here was identical with a hexapeptide sequence found only once in the amino acid sequence for the chicken intestinal epithelial cell MHC derived by

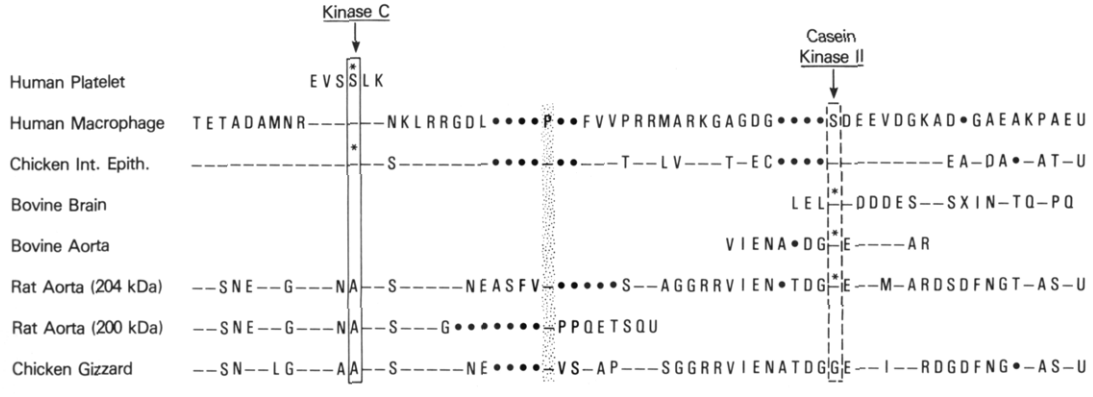

FIGURE 4: Amino acid sequence of the tryptic phosphopeptide isolated from human platelets. The solid box highlights the analogous residues in other vertebrate nonmuscle and smooth muscle MHCs. The dashed box denotes the same for the serine residues previously shown to be phosphorylated by casein kinase II (Murakami et al., 1990; Kelley & Adelstein, 1990). A dash indicates that the residue is identical with the sequence determined for human platelet or macrophage, and a dot indicates a gap inserted for the sake of aligning conserved residues. Human macrophages (Saez et al., 1990); chicken intestinal epithelium (Shohet et al., 1989); bovine brain (Murakami et al., 1990); bovine aorta (Kelley & Adelstein, 1990); rat aorta (204 and 200 kDa) (Babij & Periasamy, 1989); embryonic chicken gizzard (Yanagisawa et al., 1987). A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met, N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; X, unknown. U indicates the presence of a stop codon in the cDNA sequence at the carboxyl terminus of the protein. The proline residue that breaks the putative α-helical coiled coil is shaded. An asterisk (*) indicates that the serine residue has been shown to be phosphorylated.

cDNA cloning (Shohet et al., 1989; see Figure 4). The same sequence can also be found in the human macrophage MHC also derived by cDNA cloning (Saez et al., 1990). The phosphorylated site occurs within the predicted α-helix of the myosin rod. It is 11 amino acids amino terminal to the proline which breaks the α-helix and 45 amino acids from the carboxyl terminus (see Figure 4).

Discussion

Figure 4 shows a comparison of the amino acids in the area of the serine phosphorylated by protein kinase C for two vertebrate nonmuscle MHCs (Shohet et al., 1989; Saez et al., 1990), the 204- and 200-kDa MHC from rat aorta smooth muscle (Babij & Periasamy, 1989), and the 204-kDa smooth muscle MHC from embryonic chicken gizzard (Yanagisawa et al., 1987). The sequence of the hexapeptide isolated from human platelets, reported here, is the topmost sequence. Although the amino acids are conserved in the area of and immediately around the tryptic peptide, all three smooth muscle MHCs contain either asparagine or alanine in place of the two serine residues present in the nonmuscle MHCs. The same substitution is seen in rabbit uterine smooth muscle MHCs (Nagai et al., 1989). These findings suggest that vertebrate nonmuscle MHCs differ from smooth muscle MHCs in their ability to serve as a substrate for protein kinase C and explain the lack of phosphorylation seen for the smooth muscle MHC both in vitro (C. Kelley, NHLBI, unpublished results) and in situ (Kamm et al., 1989). This also suggests that a distinct form of regulation might play a role in affecting the structure/function of nonmuscle but not smooth muscle MHCs. Recently, the complete derived amino acid sequence of the *Drosophila* nonmuscle MHC was published (Ketcham et al., 1990). Inspection of the tail portion of this sequence reveals a serine residue, just amino terminal to the globular tail region that occupies a similar position to the serine residue in the human platelet MHC phosphorylated by protein kinase C. In contast to vertebrate nonmuscle MHCs, the residue amino terminal to the phosphorylated serine residue is an asparagine, not another serine.

The location of the phosphorylated serine has some, but not all, of the characteristics of a consensus sequence for protein kinase C (House et al., 1987). Basic residues on both sides of the phosphorylation site can have an influence on the kinetics of phosphorylation and site specificity of protein kinase C. The site phosphorylated in the nonmuscle MHC is four residues carboxyl terminal to an arginine residue and two residues amino terminal to the basic rich sequence -KXKLRR (where X is either an asparagine or serine residue). Although there is one additional residue between the phosphoserine and the arginine on the amino-terminal side, compared to a number of protein kinase C substrates, there is a significant contribution of basic residues on the carboxyl-terminal side. Perhaps this explains why only the carboxyl-terminal serine of the two serines present in the hexapeptide we isolated was found to be phosphorylated.

Recent work has suggested that there are at least two forms of the nonmuscle MHC present in most vertebrate nonmuscle cells and tissues (Katsuragawa et al., 1989; Kawamoto & Adelstein, 1991). Chicken intestinal epithelial cells are thought to contain only a single isoform of the nonmuscle MHC, which we refer to as MHC-A. To date, no sequence is available for MHC-B in the region known to be phosphorylated by protein kinase C, so it is not known if the protein kinase C site is preserved in this form of the MHC.

Figure 4 also highlights the serine residue in the MHC that has previously been shown to be a substrate for casein kinase II. A serine residue followed by multiple acidic residues appears to be required for casein kinase II recognition (Meggio et al., 1984; Marchiori et al., 1988), and the phosphorylatable serine has been shown to be present in both bovine brain MHC (a nonmuscle MHC; Murakami et al., 1990) and bovine aortic 204-kDa MHC (a smooth muscle MHC; Kelley & Adelstein, 1990). Interestingly, the 204-kDa embryonic chicken gizzard MHC contains neither a site for protein kinase C nor one for casein kinase II (see Figure 4).

The function of MHC phosphorylation by protein kinase C is unknown. The finding that the stoichiometry of phosphorylation of the MHC at this site increases from 0 to 1.0 mol of phosphate/mol of MHC following receptor aggregation in RBL-2H3 cells suggests that this phosphorylation may have physiologic relevance (Ludowyke et al., 1989). The location of the phosphorylation site suggests that it might play a role in filament stability, similar to the role that MHC phosphorylation appears to play in lower eukaryotic cells such as *Acanthamoeba* and *Dictyostelium* myosins (Atkinson et al., 1989; Sinard & Pollard, 1989). Recently, it has been demonstrated, by using antibodies to the nonmuscle MHC, that there is a decrease in the concentration of myosin in the cortical area of antigen-activated RBL-2H3 cells (Spudich et al., 1990). Since previous work (see above) has shown that the MHCs in similarly activated cells undergo phosphorylation by protein kinase C, it seems reasonable to suggest that the decrease in myosin concentration in the cortical area of these cells might be due to a decrease in myosin filaments secondary to kinase C phosphorylation.

It is of note that both bovine brain nonmuscle MHC (Murakami et al., 1990) and the 204-kDa smooth muscle MHC (Kelley et al., 1988) contain sites that are phosphorylated by casein kinase II (see Figure 4). The location of these sites differs from that phosphorylated by protein kinase C in being present in the nonhelical tail portion of the MHC 16 residues carboxyl terminal to the proline that is thought to break the α-helix in the case of the 204-kDa rat aorta MHC. Neither treatment of platelets with phorbol esters nor antigenic activation of RBL-2H3 cells results in increased MHC phosphorylation at the sites phosphorylated by casein kinase II, although cultured aortic smooth muscle cells contain 0.7 mol of phosphate/mol of MHC at this site (Kawamoto & Adelstein, 1988). The exact function of these two MHC phosphorylations in regulating the properties of vertebrate cellular myosins remains to be determined.

Added in Proof

The derived amino acid sequence of MHC-B from chicken brain cDNA indicates that the same tryptic peptide phosphorylated by kinase C is present in the carboxyl-terminal region of the MHC-B rod but that the phosphorylated serine is replaced by a threonine residue (M. Takahashi and S. Kawamoto, NHLBI, unpublished data).

Acknowledgments

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Registry No. L-Ser, 56-45-1; protein kinase, 9026-43-1.

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