

379
N812
NO, 356

APPLICATION OF SYNTHETIC PEPTIDES AS SUBSTRATES FOR
REVERSIBLE PHOSPHORYLATION

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

BY

Imad Kazem Abukhalaf, B.S., M.S.

Denton, Texas

August, 1992

379
N812
NO, 356

APPLICATION OF SYNTHETIC PEPTIDES AS SUBSTRATES FOR
REVERSIBLE PHOSPHORYLATION

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

BY

Imad Kazem Abukhalaf, B.S., M.S.

Denton, Texas

August, 1992

Qam

Abukhalaf, Imad Kazem, Application of Synthetic Peptides as Substrates for Reversible Phosphorylation, Doctor of Philosophy (Biochemistry), August 1992, 151 pp., 7 tables, 28 illustrations, References, 200 titles.

Two highly homologous synthetic peptides MLC(3-13) (K-R-A-K-A-K-T-T-K-K-R-G) and MLC(5-13) (A-K-A-K-T-T-K-K-R-G) corresponding to the amino terminal amino acid sequence of smooth muscle myosin light chain were utilized as substrates for protein kinase C purified from murine lymphosarcoma tumors to determine the role of the primary amino acid sequence of protein kinase C substrates in defining the lipid (phosphatidyl serine and diacylglycerol) requirements for the activation of the enzyme. Removal of the basic residues lysine and arginine from the amino terminus of MLC(3-13) did not have a significant effect on the K_a value of diacylglycerol. The binding of effector to calcium-protein kinase C appears to be random since binding of one effector did not block the binding of the other.

MLC(3-13) phosphorylated by protein kinase C and an S6-21, a synthetic peptide corresponding to the carboxy terminus of the ribosomal protein S6 and phosphorylated by an S6/H4 kinase were utilized as substrates for the catalytic subunits of phosphoprotein phosphatase 1 (PP1c) and 2A (PP2Ac) purified from murine lymphosarcoma tumors. PhosphoMLC(3-13) was more actively dephosphorylated by both PP1c and PP2Ac than phosphoS6-21. A sequence in phosphoS6-21 that is phosphorylated on two sites was not dephosphorylated by PP2Ac as opposed to the same sequence phosphorylated on one site suggesting that multiphosphorylated sequences may be resistant to PP2A-catalyzed dephosphorylation. The effects of various

inhibitors and activators of phosphoprotein phosphatases were shown to be substrate dependent. A novel procedure for the assay of phosphoprotein phosphatases is described.

The results demonstrate that synthetic peptides can be used as probes to characterize molecular aspects of reversible phosphorylation reactions.

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Ruthann Masaracchia, for the opportunity she gave me to work in her laboratory. The entire project would not have been possible without grants from the National Institutes of Health and the Robert A. Welch Foundation. Thanks to the Arab Student Aid International for giving me the opportunity to pursue my education.

I would like to express my gratitude for all the members of the laboratory for the camaraderie which made this work not only possible but frequently pleasant.

I am very grateful to my dear friend Gabi Abusada and Margie Schluter for their technical assistance in preparing this manuscript.

I wish to extend my profound gratitude to Mariana Fletcher for her continuous support especially during the final period of this project. I remember with special fondness the long hours we spent together in the laboratory when we discussed science and engaged in affective prose.

I am indebted to my brothers and sisters who were always there when I needed them. A very sincere thanks is extended to my brother Nazih who commiserated and listened. His companionship helped me through some very trying times.

Finally, I owe a large debt of gratitude to my parents for their unwavering support, guidance, love, encouragement, and understanding. It is to them I dedicate this volume.

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	vii
LIST OF ILLUSTRATIONS.....	viii
LIST OF ABBREVIATIONS.....	xi
 Chapter	
I. INTRODUCTION	1
Phosphoprotein phosphatases.....	2
1. Tyrosine phosphatases	2
2. Serine/threonine phosphoprotein phosphatases.....	3
a. Classification, Nomenclature and Characterization.....	3
b. Structure.....	5
c. Substrate specificity and regulation.....	6
Protein kinase C.....	11
1. Protein kinase C isoenzymes.....	13
a. Structure.....	13
b. Tissue distribution.....	14
c. Enzymological properties.....	15
Utilization of synthetic peptides as substrates for serine/threonine phosphoprotein phosphatases	15
1. Protein kinases.....	16
2. Phosphoprotein phosphatases.....	21
Purpose of this study	25
Hypothesis	26

	<u>Page</u>
II. MATERIALS AND METHODS.....	29
Materials.....	29
Methods.....	30
Tissue source	30
Purification of PP1c and PP2Ac.....	30
Purification of protein kinase C	33
Peptide synthesis.....	35
Phosphorylation of S6-21	36
Preparation of mixed micelles.....	36
Phosphorylation of MLC(3-13).....	37
Phosphorylation of gizzard light chains.....	37
Analysis of phosphopeptides	38
Amino acid analysis of thermolysin-digested peptides	39
Protein kinase C assay.....	39
Phospho S6-21 phosphatase assay.....	40
Phospho MLC(3-13) phosphatase assay	41
Gizzard phospholight chain phosphatase assay.....	41
Phosphorylase phosphatase assay.....	41
Other methods.....	42
III. RESULTS.....	43
Peptide synthesis.....	43
1. Protein kinase C.....	44
Rationale	44
Effector requirements.....	45
Phosphatidyl serine and diacylglycerol dependence of protein kinase C activation.....	46
2. Phosphoprotein phosphatases.....	49
Rationale	49
Peptide dephosphorylation assay	51
HPLC analysis of peptide dephosphorylation.....	54
Purification of PP1c and PP2Ac.....	57
Rates of dephosphorylation of phosphoMLC(3-13) and phosphoS6-21 by PP1c and PP2Ac.....	58
Kinetic analysis of PP1c- and PP2Ac-catalyzed dephosphorylation of phosphopeptides.....	59

	<u>Page</u>
Characterization of PP1c and PP2Ac using phosphoMLC(3-13) and phosphoS6-21 as substrates.....	61
Dose-dependent inhibition of PP1c and PP2Ac by microcystin-LR and okadaic acid.....	63
IV. DISCUSSION.....	128
REFERENCES	138

LIST OF TABLES

Table	<u>Page</u>
I. Effect of Protein Kinase C Activators on the Phosphorylation of MLC(3-13) and MLC(5-13) as Determined by the Standard Assay Procedure	65
II. Effect of Protein Kinase C Activators on the Phosphorylation of MLC(3-13) and MLC(5-13) as Determined by the Micellar Assay Procedure	66
III. Effect of the Order of Binding of Protein Kinase C to Phosphatidyl Serine and OAG	67
IV. Effect of Buffer on the Phosphatase Assay Background	68
V. Effect of Inhibitors and Activators on Phosphatase Assay Background	69
VI. Amino Acid Composition of Phosphopeptide C	70
VII. Kinetic Constants for the Dephosphorylation of PhosphoMLC(3-13) and PhosphoS6-21 Synthetic Peptides by PP1c and PP2Ac	71

LIST OF ILLUSTRATIONS

<u>Figure</u>	<u>Page</u>
1. Calcium Dependence of Protein Kinase C Activation.	72
2. Double Reciprocal Plot of OAG Dependence of Protein Kinase C Activation.	74
3. Phosphatidyl Serine Dependence of Protein Kinase C Activation.	76
4. Hill's Plot of Phosphatidyl Serine Dependence of Protein Kinase C Activation.	78
5. PP1c Activity as Determined by the P81 Paper Assay and the Ion Exchange Assay.	80
6. Effect of Substrate Concentration on Assay Background.	82
7. The Effect of Wash Time on Phosphatase Assay Background.	84
8. HPLC Analysis of MLC(3-13) Phosphorylated by Protein Kinase C.	86
9. HPLC Analysis of Partially Dephosphorylated MLC(3-13) by PP1c.	88
10. HPLC Analysis of Partially Dephosphorylated MLC(3-13) by PP2Ac.	90
11. HPLC Analysis of MLC(3-13) Completely Dephosphorylated by PP1c.	92
12. HPLC Analysis of S6-21 Phosphorylated by S6/H4 Kinase.	94
13. HPLC Analysis of Partially Dephosphorylated S6-21 by PP2Ac.	96
14. A. HPLC Analysis of Phosphopeptides Obtained by Digestion of PhosphoS6-21 with Thermolysin. (PP1c)	98

<u>Figure</u>	<u>Page</u>
15. A. HPLC Analysis of Phosphopeptides Obtained by Digestion of PhosphoS6-21 with Thermolysin. (PP2Ac)	100
16. Effect of Inhibitor-2 on the Phosphorylase Phosphatase Activity of PP1c and PP2Ac.	102
17. Rate of Dephosphorylation of PhosphoMLC(3-13) by PP1c and PP2Ac.	104
18. Rate of Dephosphorylation of PhosphoMLC(3-13) by Diluted PP1c and PP2Ac.	106
19. Rate of Dephosphorylation of Gizzard Phospholight Chains by PP1c and PP2Ac.	108
20. Rate of Dephosphorylation of PhosphoS6-21 by PP1c and PP2Ac.	110
21. Double Reciprocal Plot of Initial Velocities vs. PhosphoMLC(3-13) Concentration with PP1c.	112
22. Double Reciprocal Plot of Initial Velocities vs. PhosphoMLC(3-13) Concentration with PP2Ac.	114
23. Double Reciprocal Plot of Initial Velocities vs. PhosphoS6-21 Concentration with PP1c.	116
24. Double Reciprocal Plot of Initial Velocities vs. PhosphoS6-21 Concentration with PP2Ac.	118
25. Effect of Various Phosphatase Inhibitors and Activators on the Dephosphorylation of PhosphoMLC(3-13) by PP1c and PP2Ac.	120

<u>Figure</u>	<u>Page</u>
26. Effect of Various Phosphatase Inhibitors and Activators on the Dephosphorylation of PhosphoS6-21 by PP1c and PP2Ac.	122
27. Dose-Dependent Inhibition of PP1c and PP2Ac by Microcystin-LR.	124
28. Dose-Dependent Inhibition of PP1c and PP2Ac by Okadaic Acid.	126

LIST OF ABBREVIATIONS

ATP.....	adenosine 5'-triphosphate
BME.....	β -mercaptoethanol
cAMP.....	cyclic adenosine 3'; 5' monophosphate
cGMP.....	cyclic guanosine 3'; 5' monophosphate
CM.....	carboxy methyl
DEAE.....	diethylaminoethyl
DAG.....	diacylglycerol
DTE.....	dithioerythritol
EDTA.....	ethylenediaminetetraacetic acid
EGF.....	epidermal growth factor
eIF-2.....	eukaryotic polypeptide initiation factor
FPLC.....	fast protein liquid chromatography
G-subunit.....	regulatory subunit of phosphoprotein phosphatase 1
GLC.....	myosin light chains from gizzard muscle
HEPES.....	N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid
HPLC.....	high performance liquid chromatography
MES.....	2[N-Morpholine]ethane sulfonic acid
Microcystin-LR.....	microcystin-leucine-arginine
MLC.....	myosin light chain
MLCK.....	myosin light chain kinase
Mr.....	apparent molecular weight

O.A.....	okadaic acid
OAG.....	L- α -1-oleoyl-2-acetoxy- <i>sn</i> -3-glycerol
phosphoMLC.....	myosin light chain peptide phosphorylated by protein kinase C
phosphoS6-21	S6-21 peptide phosphorylated by S6/H4 kinase
PKA.....	cyclic AMP-dependent protein kinase
PKC	calcium/phospholipid-dependent protein kinase
PNPP.....	<i>para</i> nitrophenyl phosphate
PP1.....	phosphoprotein phosphatase 1
PP1c.....	catalytic subunit of phosphoprotein phosphatase 1
PP2Ac	catalytic subunit of phosphoprotein phosphatase 2A
PP1-G.....	glycogen-attached phosphoprotein phosphatase 1
PP1-I.....	cytosolic inactive phosphoprotein phosphatase 1
PP1-M.....	myofibril-attached phosphoprotein phosphatase 1
PMSF.....	phenylmethylsulfonyl fluoride
PS.....	phosphatidyl serine
S-200	sephacryl-200 gel filtration resin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA.....	trifluoroacetic acid

CHAPTER I

INTRODUCTION

A large number of proteins in mammalian cells contain covalently bound phosphate. While in many cases the functional significance of this modification is unclear, it is nevertheless obvious that protein phosphorylation plays an essential role in signal transduction enabling cells to regulate cellular processes through the reversible covalent modification of enzymes and other target proteins. The phosphorylation state of any protein, and thus its phosphate content, represents a net balance of the relative activities of protein kinases and phosphatases.

It has been suggested that more than 1000 protein kinases may exist (1), and several of these kinases are known to be regulated by second messengers such as cyclic AMP (cyclic AMP-dependent protein kinase) (2), cyclic GMP (cyclic GMP-dependent protein kinase) (3, 4), and Ca^{+2} (calcium/calmodulin-dependent protein kinases (5, 6).

In contrast to protein kinases which have been under intensive investigation for decades, and for which a role in signal transduction is well documented (7-19), protein phosphatases have been relatively ignored for many years. This was, in part, due to technical difficulties, since both the regulated phosphoprotein substrate and its protein kinase(s) must first be purified before a substrate for studying the dephosphorylation process can be made. Another reason for the comparatively slow progress in defining

phosphatases has been the mistaken impression that protein phosphatases were constitutively active and not regulated by the growth factors, hormones, and extracellular signals that regulate protein kinases.

This era of neglect ended following the discovery that several protein phosphatases are highly regulated and that their involvement in reversible phosphorylation/dephosphorylation cascade systems renders them key players in controlling signal transduction and many other cellular events.

Phosphoprotein phosphatases

Phosphoprotein phosphatases (EC 3.1.3.—) are ubiquitous proteins which reverse the actions of protein kinases by catalyzing the dephosphorylation of phosphoproteins to apoproteins and inorganic phosphates.



I. Tyrosine phosphatases

Phosphotyrosine is a rare phosphoamino acid, but its level increases dramatically in cells stimulated by growth factors or transformed by certain RNA tumor viruses (20-22). The receptors for these growth factors and the proteins encoded in the transforming gene (oncogene) of these viruses have been identified as protein tyrosine kinases. Under normal homeostatic conditions, phosphotyrosine-containing proteins do not accumulate, due to the presence of phosphotyrosine phosphatases that reverse the action of tyrosine kinases (23).

A number of cytoplasmic and membrane bound tyrosine protein phosphatases have been identified (24-26). These enzymes most likely play a role in the control of signal transduction by the tyrosine kinase activities of receptors for growth factors, peptide hormones, and oncogene products. Phosphotyrosine phosphatases are inhibited by micromolar concentrations of either Zn^{+2} or orthovanadate and show maximal activity in the presence of the divalent cation chelator EDTA (27). It has been indicated that soluble as well as particulate phosphotyrosyl protein phosphatases dephosphorylate specific sites of the human insulin receptor tyrosine kinase which in turn lead to its deactivation (28). The discovery that the receptor-like transmembrane protein CD45 (also known as leukocyte common antigen) is homologous to a tyrosine phosphatase from human placenta (29), and the confirmation that CD45 possesses intrinsic phosphatase activity (30, 31) introduced the concept for a new signal transduction mechanism involving the activation of a phosphatase receptor.

2. Serine/Threonine Phosphoprotein Phosphatases

a. Classification, Nomenclature, and Characterization

Ingebritsen and Cohen (32) identified two major classes of phosphoprotein phosphatases that are responsible for virtually all the dephosphorylation activity towards a number of phosphoserine/threonine-containing proteins involved in various regulation reactions in rat liver and rabbit liver and skeletal muscles. For example, glycogen metabolism (33-35), glycolysis/gluconeogenesis, fatty acid synthesis, cholesterol synthesis (36, 37),

and protein synthesis (38-46) appear to be regulated by phosphoprotein phosphatases as well as protein kinases. These phosphatases were designated type 1 (PP1) and type 2 (PP2) protein phosphatases (32). The type 2 enzymes were further subdivided into three groups, designated PP2A, PP2B, and PP2C (47).

This classification of protein phosphatases is based on their molecular and catalytic properties. The two classes of phosphoserine/threonine protein phosphatases can be distinguished by their reactivity towards phosphorylase kinase and by their sensitivity to certain activators and inhibitors. PP1 is potently inhibited by the thermostable protein inhibitors 1 and 2 (I-1 and I-2 respectively) and dephosphorylates the β -subunit of phosphorylase kinase specifically, whereas PP2 is unaffected by the inhibitor proteins and preferentially dephosphorylates the α -subunit of phosphorylase kinase (32, 47). PP1 and PP2A are distinguished from PP2B and PP2C on the basis of their substrate reactivities as well. PP2B and PP2C possess negligible activity towards phosphorylase *a* (48, 49).

The three type 2 phosphoprotein phosphatases can be distinguished in a number of ways. Differential requirements for divalent cations and their response to some natural products, like okadaic acid and microcystin-LR, are most dramatic. PP2A does not have an absolute requirement for divalent cations, whereas PP2B is a Ca^{+2} -dependent, calmodulin-stimulated enzyme, and PP2C is dependent on Mg^{+2} (47).

Recently, okadaic acid, a non-phorbol ester tumor promoter produced by marine dinoflagellates, has been shown to be a potent inhibitor of PP2A ($\text{IC}_{50} = 10\text{-}20\text{ nM}$) although it also effectively inhibits both PP1 and PP2B ($\text{IC}_{50} = 1\text{ }\mu\text{M}$

and 50 μ M, respectively, whereas PP2C is not affected by this toxin (50-57). In addition, microcystin-LR, a hepatotoxic cyanobacterial cyclic heptapeptide toxin, is an even more potent inhibitor than okadaic acid. K_i values less than 0.1 nM are observed with PP1 and PP2A, whereas PP2B is inhibited 1000-fold less potently by this peptide (58-62).

More recently, the antibiotic tautomycin was found to be a potent inhibitor of phosphoprotein phosphatases. It was equally effective for the type 1 and type 2A enzymes. For the catalytic subunits of PP1 and PP2A (PP1c and PP2Ac respectively), the IC_{50} was 22 to 32 nM (63).

b. Structure

Recent progress concerning serine/threonine protein phosphatases has come largely from molecular cloning and cDNAs for the catalytic subunits of these enzymes (64, 65).

Analysis of the predicted primary structure of the two phosphatase classes indicates substantial structural homology between PP1, PP2A, and PP2B (66-68). Peptide sequencing and cDNA cloning have failed to reveal any homology between PP1/PP2A and PP2C, which indicates that the PP2C isoforms represent a quite distinct protein phosphatase gene family (69-70). Complementary DNA cloning and analysis of the predicted primary structure of PP1 from mammalian tissues (71, 72), *Drosophila* (73), yeast (74), and *Aspergillus* (75), and PP2A from mammalian tissues (76) has revealed extreme conservation of these enzymes throughout evolution. Moreover, the catalytic subunits of various PP1 and PP2 phosphoprotein phosphatases

designated PP1c and PP2Ac, respectively, provide a striking example of the conservation of this class of protein phosphatases through the years (65). Furthermore, PP1, PP2A, and PP2C were identified in plants and their characteristics were found to be virtually indistinguishable from the corresponding enzymes in mammalian tissues (77).

c. Substrate specificity and regulation

Phosphorylase *a* is the classic substrate for phosphoprotein phosphatases, for it was the first enzyme shown to be reversibly phosphorylated (48). Both PP1 and PP2 dephosphorylate the lone phosphoserine at residue 14 in phosphorylase *a* (48). Although the characterization of different phosphoprotein phosphatases in terms of their susceptibility to various inhibitors and activators was based on the phosphatases' reactivities with phosphorylase *a*, the phosphorylase phosphatase activity of PP1 and PP2A constitutes only 27 and 17% of that with β and α subunits of phosphorylase kinase respectively (32). In addition, p-nitrophenylphosphate (PNPP), a non-protein phosphoester, is dephosphorylated by both PP1 and PP2A, with PP1 activity being 10% that of PP2A (78).

The catalytic subunits of the two major phosphoprotein phosphatases (PP1 and PP2A) can be separated from the regulatory subunits of the enzymes. Endogenous proteases and/or exogenous trypsin, ethanol or acetone dissociate the catalytic subunits which are resistant to all the mentioned reagents, thereby increasing the phosphatase activity (79, 80).

Both enzymes PP1c and PP2Ac catalyze the dephosphorylation of histone H1 phosphorylated by cAMP-dependent protein kinase (81). In contrast, when histone H1 phosphorylated by protein kinase C is used as a substrate, PP1 activity decreases 20-fold whereas PP2Ac activity increases 60-fold. However, this substrate specificity is observed under defined assay conditions and whether it is physiologically significant is unknown. Whether it is phosphorylated by cAMP-dependent protein kinase or protein kinase C, histone H1 is not dephosphorylated by either PP2B or PP2C (81).

In contrast to PP2A which is mainly cytosolic, various forms of PP1 are associated with cellular organelles and it is believed that the interaction of PP1 with various particulate substrates is mediated by specific regulatory (targeting) subunits (65). In mammalian skeletal muscle, PP1 is mainly associated with glycogen particles, myofibrils, and the sarcoplasmic reticulum through its regulatory subunits (65). The PP1 form that is glycogen-associated (PP1-G), is a heterodimer composed of a catalytic subunit (C-subunit) complexed to a glycogen-binding subunit (G-subunit) that is responsible for association with glycogen (82-84). PP1-G is 5-10-fold more active than the free C-subunit in dephosphorylating the rate-limiting enzymes of glycogenolysis (phosphorylase *a*) and glycogen synthesis (glycogen synthase), but enhanced activity is only observed when both PP1-G and its substrates are bound to glycogen (65). PP1-G has the same activity as the free catalytic subunit toward substrates which do not interact with glycogen such as myosin light chains (65). These studies suggested the concept of targeting subunits as a mechanism for selectively enhancing PP1 activity toward certain substrates and for directing it to particular subcellular locations.

Phosphoprotein phosphatase type 1 is the major enzyme acting on phosphorylase *a* and glycogen synthase in skeletal muscles. It is also the principal enzyme in dephosphorylating the β -subunit of phosphorylase kinase, thus responsible for the inactivation of glycogenolysis and stimulation of glycogen synthesis (85). Depending on the form of the enzyme and the tissue, PP1 regulation is controlled by both Ca^{+2} and cAMP. One mechanism is through the phosphorylation of the targeting subunits such as the G-subunit. Skeletal muscle PP1 G-subunit is phosphorylated *in vitro* by PKA on two serine residues, termed site 1 and site 2 (82, 83), and *in vivo* it is phosphorylated on the same sites in response to epinephrine (86). The phosphorylation of site 2 by PKA triggers the dissociation of C-subunit from G-subunit and the translocation of the former from the glycogen particles to the cytosol (48, 87), where it is likely to be inactivated by inhibitor-1 (88), a potent inhibitor of the C-subunit when phosphorylated by PKA. The dissociation of the C-subunit from the G-subunit is a mechanism for selectively inhibiting the dephosphorylation of the glycogen metabolizing enzymes such as phosphorylase *a* and glycogen synthase (9). This explains how levels of phosphorylase *a* are kept high in resting muscle in response to epinephrine and how epinephrine increases the phosphorylation of glycogen synthase (89). The insulin-stimulated phosphorylation of the G-subunit at site 1 by PKA has no effect on the rate of dephosphorylation of phosphorylase *a* by PP1-G in skeletal muscle (90).

The major phosphoprotein phosphatases which dephosphorylate the G-subunit and inhibitor-1 appear to be PP2A and PP2B (48, 91). PP1 can therefore be controlled indirectly by substances that regulate these other protein phosphatases. The dephosphorylation of the G-subunit and inhibitor-1 by

PP2B (Ca^{+2} /calmodulin-dependent) are potential mechanisms by which Ca^{+2} can activate PP1 and attenuate the action of cAMP (92).

The PP1 form that is associated with the sarcoplasmic reticulum is PP1-G, suggesting that the G subunit may contain a domain for interaction with sarcoplasmic reticulum as well as regions that interact with glycogen and with the catalytic subunit (48). The form of PP1 associated with cardiac and skeletal muscle myofibrils is distinct from PP1-G. It possesses high activity towards myosin and is referred to as PP1-M. PP1-M catalyzes the dephosphorylation of myosin light chain phosphorylated by myosin light chain kinase (MLCK) in skeletal muscle, cardiac muscle, and isolated myosin light chains (93, 94). Data suggest that PP1-M is composed of a catalytic subunit that is similar to C-subunit of PP1-G complexed to another subunit which binds myosin, thus responsible for stimulating the activity towards this substrate (93). Moreover, it has been reported that myosin in gizzard muscle is dephosphorylated by a phosphatase with higher molecular weight than PP1-M (95). Substrate specificity studies suggest that this enzyme also consists of a catalytic subunit and a myosin binding subunit. These enzymes are likely to be the principal myosin and myosin light chain phosphatases. PP2A from either cardiac or skeletal muscle was essentially inactive against myosin whereas it showed substantial activity against the isolated myosin light chain (94).

The inactive cytosolic form of PP1 (PP1-I) is present in many cells. It is a complex of a catalytic subunit, which is similar if not identical to the catalytic subunits of the other forms of PP1, and inhibitor-2. It can be activated *in vitro* through the phosphorylation of inhibitor-2 (48, 96).

S6, the major phosphoprotein of the ribosomal subunit 40S, is phosphorylated on multiple sites by more than one S6 kinase in a variety of cells in response to insulin and other growth factors that stimulate protein synthesis. Unlike PP2A, a significant portion of PP1 activity in rabbit reticulocyte lysates is associated with ribosomes (97), suggesting the occurrence of ribosome-binding regulatory subunit. About 80% of S6 phosphatase activity in *Xenopus* oocyte extracts was diminished when I-2 was added to the extract (40). Moreover, S6 phosphorylation was increased about 3-fold upon the microinjection of I-2 into intact *Xenopus* oocytes (40). Similarly, PP1 has been reported to be the major S6 phosphatase in mouse 3T3 cells (98). However, PP2A plays an important role in the regulation of S6 phosphorylation, for it is the major phosphatase activity in mouse 3T3 cells that inactivates a mitogen-stimulated S6 kinase that phosphorylates S6 (39, 99). In addition, S6 kinase II from *Xenopus* is dephosphorylated and thus inactivated *in vitro* by both PP1 and PP2, but it is reactivated by phosphorylation by MAP-2 kinase which is itself inactivated by PP2A (100).

The phosphorylation of the α -subunit of the initiation factor eIF-2 on a single serine residue inhibits protein synthesis in reticulocytes (101). Although PP1 and PP2A efficiently dephosphorylated the α -subunit of eIF-2 *in vitro*, it seems that PP1 is the principal phosphatase that dephosphorylates the α -subunit of eIF-2 *in vivo* because, unlike PP2A, it is specifically associated with ribosomes, and the addition of I-2 to rabbit reticulocytes lysates increases the phosphorylation of eIF-2 and inhibits the polypeptide chain initiation (102). Also, PP2A may play a key role in the regulation of eIF-2

phosphorylation/dephosphorylation cycle, for it is the principal phosphatase that inactivates the kinase that phosphorylates the α -subunit of eIF-2 (103).

Phosphoprotein phosphatase 2A has high activity toward L-pyruvate kinase, ATP-citrate lyase, and acetyl-CoA carboxylase in rat liver, suggesting an important role in the regulation of glycolysis/gluconeogenesis and fatty acid synthesis (48, 104). In addition, it has been reported that PP2A may be the most important inhibitor-1 phosphatase, suggesting that it may control glycogen metabolism indirectly by stimulating PP1 through this mechanism (105).

Polycation polypeptides such as poly L-lysine, histone H1, histone H3, and protamine have been shown to markedly activate PP2A (104). The exact mechanism of activation is unclear but is considered to occur primarily via the interaction of these effectors with the catalytic subunit and does not involve the dissociation of the enzyme complex (104). The effect of polycations vary with different substrates, indicating that substrate interaction may also occur (103).

PP2A has also been found to dephosphorylate proteins which are themselves involved in signal transduction pathways. In addition to its potential role in regulating PP1 activity in cells (105, 106), a role in regulating protein kinases has also been demonstrated.

Protein Kinase C

Protein kinase C (PKC) is a ubiquitous calcium/phospholipid-dependent protein kinase. It is a serine/threonine protein kinase and is involved in a number of signal transduction mechanisms for several growth factors and

hormones (20, 107, 108). It has been purified to homogeneity from a number of tissues (109, 110).

The stimulation of inositol phospholipid metabolism causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃). Diacylglycerol interacts with protein kinase C causing its activation, possible translocation to the plasma membrane, and the phosphorylation of various target proteins within the cell (111-114). However, IP₃ binds to an intracellular receptor on the smooth endoplasmic reticulum. Once the receptor is occupied, calcium ions are released to the cytosol resulting in a transient increase in calcium concentration (115). The exact mechanism of calcium release is unknown. Although the hydrolysis of inositol phospholipids was thought to be the sole mechanism leading to the activation of protein kinase C, recent studies suggest that there are several additional signal pathways to provide diacylglycerol which is needed for protein kinase C activation. For example, phosphatidyl choline may also be hydrolyzed to produce DAG at a relatively later stage in cellular responses (116). Phorbol esters and other tumor promoters with structural similarities to DAG can activate protein kinase C by binding to the DAG binding site. In addition, it has been reported that some hormonal stimulation can lead to a rapid increase of inositol 1,3,4,5 tetraphosphate (IP₄) concentrations (117-118). Although the IP₄ metabolism kinetics suggest that it may play a role in modulating intracellular events, IP₄ does not mobilize Ca⁺² from intracellular stores (119).

In addition to a number of proteins that protein kinase C phosphorylates *in vitro* (16), myosin light chain, S6 protein on the 40S ribosome, EGF receptor,

the data suggest that substrate and enzyme may determine effector requirements.

1. Protein Kinase C isoenzymes

Molecular cloning and enzymological analysis has revealed the existence of multiple mammalian protein kinase C isoenzymes with closely related structures. Initially, four cDNA clones, which encode α , β I, β II, and γ subspecies, were found. Subsequently, additional cDNA clones, encoding at least five other isoenzymes designated δ , ϵ , ζ , η , and L, have been isolated from a rat brain library (129-139).

a. Structure

Protein kinase C isoenzymes are all composed of a single polypeptide chain, with isoenzymes α , β I, β II, and γ having four conserved and five variable regions (140). The β I and β II isoenzymes differ from each other only in the fifth variable region (18). Although the isoenzymes δ , ϵ , ζ , η and L lack the second conserved region, all isoenzymes have the same molecular mass of approximately 77,000 with the exception of PKC- ζ which is smaller (M_r = 67,700) (16). The amino terminal half is the regulatory domain. The first conserved region of each isoenzyme, with the exception of PKC- ζ , contains a cystein-rich tandem repeat sequence that might be essential for binding DAG and phorbol esters (15, 113, 141, 142). The second conserved region which is absent from the δ , ϵ , ζ , η and L isoenzymes is required for the Ca^{+2} sensitivity of the enzyme (16). The carboxy terminal half is the catalytic domain which

conserved region, all isoenzymes have the same molecular mass of approximately 77,000 with the exception of PKC- ζ which is smaller ($M_r = 67,700$) (16). The amino terminal half is the regulatory domain. The first conserved region of each isoenzyme, with the exception of PKC- ζ , contains a cystein-rich tandem repeat sequence that might be essential for binding DAG and phorbol esters (15, 113, 141, 142). The second conserved region which is absent from the δ , ϵ , ζ , η and L isoenzymes is required for the Ca^{+2} sensitivity of the enzyme (16). The carboxy terminal half is the catalytic domain which contains the protein substrate binding site and the ATP-binding sequence (G-X-G-X-X-G---K) in the third conserved region (16). It has been reported that the regulatory domain and the kinase domain are cleaved at specific sites(s) by Ca^{+2} -dependent protease (calpain), a mechanism by which the degradation of protein kinase C is initiated (18).

b. Tissue distribution

Using a combination of biochemical and immunohistochemical techniques, the relative activities and the cell type and tissue distribution of different protein kinase C isoenzymes have recently been examined (19, 112, 113, 141, 143-145). PKC- γ is expressed only in the central nervous tissue and is not found in any other tissue or cell type. On the other hand, PKC- βI and βII are present mainly in the brain and at lower levels in other tissues. In contrast, PKC- α is widely distributed in many tissues and cell types. In general, each cell type contains more than one protein kinase C isoenzyme and subcellular distribution is dependent upon the biochemical functions of the cell.

c. Enzymological properties

Different protein kinase C isoenzymes exhibit some differences in enzymatic properties. These isoenzymes can be grouped into two categories based on Ca^{+2} requirements for activation: Ca^{+2} -dependent and Ca^{+2} -independent. Protein kinase C isoenzymes α , βI , βII , and γ are Ca^{+2} and phospholipid-dependent kinase, whereas the δ , ϵ , ζ , and η isoenzymes are calcium-independent, phospholipid-stimulated kinases (16).

The βI and βII isoenzymes have almost identical kinetics and catalytic properties and can be distinguished from each other by immunochemical techniques only. PKC- βI and βII are substantially active without added Ca^{+2} in the presence of diacylglycerol and phosphatidyl serine (16).

Utilization of synthetic peptides as substrates for serine/threonine kinases and serine/threonine phosphoprotein phosphatases

Utilization of synthetic peptides for enzymological studies of reversible phosphorylation has many advantages over protein substrates. For example, peptides corresponding to specific sequences in physiological substrates are easy to synthesize. Also, the sequences of the peptides are conveniently varied in order to study the recognition site(s) and/or the specificity determinants of protein kinases and phosphatases. Stoichiometry and site(s) of phosphorylation/dephosphorylation are easily determined by direct sequencing, and concerns about substrate denaturation during analyses are avoided. Also, depending on recognition sequences determined by the

utilization of synthetic peptides, several phosphorylation sites in proteins were determined from the cDNA sequences of those proteins. Finally, peptide synthesis or obtaining custom-made peptides is relatively inexpensive.

1. Protein kinases

Synthetic peptides have been used extensively to facilitate the analysis and determine the substrate specificity of several protein kinases. The specificity determinants for cyclic AMP-dependent protein kinase (146), protein kinase C (147, 148), an S6/H4 kinase (149, 150), calcium-dependent protein kinases (151, 152) and cyclic GMP-dependent protein kinase (154) have been investigated using synthetic peptides derived from unique protein kinase substrates.

Initial studies using synthetic peptides to determine the substrate specificity of the catalytic subunit of rabbit skeletal muscle PKA used the synthetic peptide R-G-Y-S-L-G corresponding to a phosphorylation site sequence in chicken egg white lysozyme (146). This peptide was phosphorylated by the enzyme with apparent K_m of 4.2 mM and a V_{max} of 16.4 $\mu\text{mol/min per mg}$ which is approximately 6-fold higher than that of the protein substrate. Replacement of the amino terminal arginine with glycine, histidine or lysine resulted in a significant increase in the apparent K_m (12.4, 13.4 and 14.7 mM respectively) and a dramatic reduction in the V_{max} (0.41, 0.95 and 0.86 $\mu\text{mol/min-mg}$ respectively). These results support the concept that arginine plays an important role in determining the substrate specificity of this protein kinase (146). Further studies on the substrate specificity of the PKA

catalytic subunit conducted with the synthetic peptide L-R-R-A-S-L-G (later known as Kemptide) which corresponds to a phosphorylation site sequence in liver pyruvate kinase (153). The peptide was phosphorylated by the enzyme with an apparent K_m and V_{max} of 16 μM and 20.2 $\mu mol/min-mg$ respectively. Replacement of either of the two arginine residues with alanine resulted in at least 300-fold increase in the apparent K_m values. The presence of arginine residues in proximity to the phosphorylation site was further proven to be important since the substitution of either arginine residue with other basic amino acids such as lysine, histidine or homoarginine resulted in a significant increase in the apparent K_m values (830, 878 and 395 μM respectively). Substitution of the serine with threonine resulted in approximately 35-fold higher K_m value (590 μM) than that of the parent peptide. The observed V_{max} was approximately 25% of that of the parent peptide. Truncation of the peptide by one or two amino acid residues for either terminus did not have a significant effect on the kinetic constants of the phosphorylation (154). To investigate whether all protein kinases have the same substrate specificity or not, the synthetic decapeptide K-R-K-Q-I-S-V-R-G-L which corresponds to the phosphorylation site sequence of glycogen phosphorylase has been tested as a substrate for the catalytic subunit of PKA and phosphorylase kinase (154). The peptide was phosphorylated by both enzymes. However, with PKA, the apparent K_m (3900 μM) was 4-fold higher than that with phosphorylase kinase (900 μM). Substitution of alanine for the first arginine from the amino terminal had no effect on the kinetic constants when the peptide was used as a substrate for phosphorylase kinase. On the other hand, the V_{max} was reduced approximately 100-fold by this analog peptide when it was used as a substrate

for PKA. Substitution of the other arginine in the peptide with alanine dramatically reduced the apparent K_m value ($36 \mu\text{M}$) when this analog was used as a substrate for PKA. Also, it was a poor substrate for phosphorylase kinase (apparent $K_m = 2500 \mu\text{M}$). These results confirmed that both PKA and phosphorylase kinase require arginine residues in proximity to the phosphorylation site. However, their substrate specificity differ in that phosphorylase kinase requires an arginine on the carboxyl terminal side of the phosphorylation site, whereas PKA requires arginine on the amino terminal side of the phosphorylation site (154).

Substrate specificity determinants for skeletal muscle myosin light chain kinases using synthetic peptides have been investigated (151). Synthetic peptides derived from the skeletal muscle myosin P-light chain (P-K-K-A-K-R-R-A-A-E-G-S-S-N-V-F-S) and smooth muscle myosin P-light chain (S-S-K-R-A-K-A-K-T-T-K-K-R-P-Q-R-A-T-S-N-V-F-S) were phosphorylated by myosin light chain kinases purified from rabbit and chicken skeletal muscles. Both enzymes had similar phosphorylation kinetics with synthetic peptide substrates with average V_{max} of $0.9 \mu\text{mol/min-mg}$ and an apparent K_m of $2.3 \mu\text{M}$ when the skeletal muscle myosin light chain-derived peptide was used as a substrate. With the smooth muscle-derived peptide, the observed V_{max} and apparent K_m were $27 \mu\text{mol/min-mg}$ and $1.4 \mu\text{M}$ respectively. When the two lysine residues closest to the amino terminus in the skeletal muscle-derived peptide were substituted with two alanine residues, the K_m increased by approximately 50-fold. Substitution of the two arginine residues with two alanine residues resulted in 70-fold increase in K_m . Substitution of glutamic acid with arginine in the parent peptide resulted in a 3-fold decrease in K_m value. Truncation of the

tremendous increase in K_m . These results suggested that basic residues in proximity to the phosphorylation site are important substrate determinants for skeletal muscle myosin light chain kinases (151). Similar studies were conducted with other synthetic peptides with smooth muscle myosin light chain kinases. These studies indicated that basic residues in the vicinity of the phosphorylation site are the primary determinants of substrate specificity for these enzymes (151, 155-158). In addition, smooth muscle myosin light chain kinases do not phosphorylate skeletal muscle myosin light chain kinase substrates, nor do they phosphorylate substrates specific for PKA or phosphorylase kinase (151).

Due to the role of protein kinase C as a pivotal enzyme in many signal transduction pathways, protein kinase C substrate specificity has been under intensive investigation. For this purpose, synthetic peptides derived from several of the protein substrates of the enzyme have been utilized. It has been reported that the ribosomal peptide S6 (229-239) containing the sequence R-R-L-S-S-L-R-A is an excellent substrate for protein kinase C (147). Moreover, it has been shown that the serine/threonine-containing peptide derived from smooth muscle myosin, MLC(1-13), is phosphorylated by purified protein kinase C, and the removal of the two serine residues from the amino terminus of the peptide did not affect its rate of phosphorylation by the enzyme. In addition, other protein kinases such as PKA, cGMP-dependent protein kinase, S6/H4 kinase, MLCK, and calcium/calmodulin-dependent protein kinase II were virtually inactive with the synthetic peptide or its analogs (148). This and many other studies on protein kinase C concluded that the presence of basic residues

has been shown that the serine/threonine-containing peptide derived from smooth muscle myosin, MLC(1-13), is phosphorylated by purified protein kinase C, and the removal of the two serine residues from the amino terminus of the peptide did not affect its rate of phosphorylation by the enzyme. In addition, other protein kinases such as PKA, cGMP-dependent protein kinase, S6/H4 kinase, MLCK, and calcium/calmodulin-dependent protein kinase II were virtually inactive with the synthetic peptide or its analogs (148). This and many other studies on protein kinase C concluded that the presence of basic residues such as arginine in proximity to the phosphorylation site are important specificity determinants for protein kinase C (147, 148, 159-165).

Studies with S6/H4 kinase and PKA have shown that the synthetic peptide (V-K-R-I-S-G-L) derived from histone H4 sequence is phosphorylated by both enzymes (166). However, when phosphorylated by PKA, the apparent K_m value was approximately 37-fold higher than that of H4 protein kinase. Substitution of lysine with arginine resulted in approximately 7-fold increase in K_m when phosphorylated by H4 kinase and 15-fold decrease when phosphorylated by PKA. Translocation of the lysine-arginine sequence to a position that is one residue further from the phosphorylation site markedly decreased the ability of H4 protein kinase to phosphorylate the peptide. In addition, replacing the arginine closer to the amino terminus of the peptide with lysine, resulted in dramatic decrease in the apparent K_m of the peptide when phosphorylated by H4-kinase and significant increase when phosphorylated by PKA. These studies suggested that, for optimal activity, S6/H4 kinase requires the presence of a lysine-arginine sequence at the amino terminal side one residue away from the phosphorylation site, and that PKA prefers the arginine-

arginine sequence over lysine-arginine sequence. In addition, these results confirm that although both enzymes require the presence of basic residues for optimal activity, their specificity determinants are not identical (166).

The above studies, along with similar others, established that the kinetic constants observed with synthetic peptides are comparable to those observed with intact protein substrates. They also established the requirement for and the optimal location of specific basic residues near the phosphorylation site on several protein kinase substrates. For example, the minimal requirements for PKA optimal activity was reported to be R-R-X-S or R-X-K-R-X-X-S-K, and that for S6 kinase II is R-X-X-S. The recognition sequence for skeletal as well as smooth muscle myosin light chain kinases was reported to be K-K-R-X-X-R-X-X-S and that of S6/H4 kinase is L-R-X-S. The recognition sequence for protein kinase C (α , β and γ) was suggested to be R-X-X-S-X-R (167).

In general, these studies demonstrate that the primary amino acid sequence in the protein substrates of protein kinases is an important determinant for protein kinase recognition. A higher ordered structure of the substrates is not necessary for phosphorylation to occur, although additional contributions from additional domains may refine the enzyme kinetics.

Collectively, these studies confirm that employment of synthetic peptides proved to be useful in the determination of substrate specificity of protein kinases.

as substrates is that they are typically phosphorylated at a low stoichiometry at several different residues in the polypeptide chain. Such heterogeneous preparations can only be used in a narrow range, limiting their utilization as phosphatase substrates. Another reason for the limited information on the substrate specificity of phosphoprotein phosphatases is the lack of a simple and reliable assay procedure.

A few preliminary attempts have been made to apply the synthetic peptide strategy to the determination of phosphatase substrate specificity. Early studies of PP2Ac isolated from rat liver revealed that phosphopeptides phosphorylated by PKA corresponding to the phosphorylation sites of rat liver pyruvate kinase and the β subunit of rabbit muscle phosphorylase *b* kinase (L-R-R-A-S-V-A-E-L and A-R-T-K-R-S-G-S-V-Y-E-P-L-K respectively) are dephosphorylated by the enzyme. The observed V_{max} and apparent K_m for L-R-R-A-S-V-A-E-L peptide, which is similar but not identical to Kemptide, were 3.3 nmol/min-mg and 110 μ M respectively. For the same peptide with Q substituted for E, the V_{max} and K_m were 4.2 nmol/min-mg and 60 μ M respectively. The truncation of the peptide by two amino acids from the carboxyl terminal side resulted in a tremendous increase in apparent K_m (500 μ M) and a significant decrease in V_{max} (1 nmol/min-mg). Additional truncations from the amino terminal side resulted in a decrease in K_m value with little or no effect on V_{max} . The observed V_{max} and apparent K_m for A-R-T-K-R-S-G-S-V-Y-E-P-L-K were comparable to those of the other peptide (22 nmol/min per mg and 110 μ M respectively). Truncation of the peptide by four amino acids from the carboxyl terminal side did not have any effect on V_{max} whereas apparent K_m increased by 2-fold. Truncation of the peptide from the amino terminal side by

R-R-A-S-V-A-E-L peptide, which is similar but not identical to Kemptide, were 3.3 nmol/min-mg and 110 μ M respectively. For the same peptide with Q substituted for E, the V_{max} and K_m were 4.2 nmol/min-mg and 60 μ M respectively. The truncation of the peptide by two amino acids from the carboxyl terminal side resulted in a tremendous increase in apparent K_m (500 μ M) and a significant decrease in V_{max} (1 nmol/min-mg). Additional truncations from the amino terminal side resulted in a decrease in K_m value with little or no effect on V_{max} . The observed V_{max} and apparent K_m for A-R-T-K-R-S-G-S-V-Y-E-P-L-K were comparable to those of the other peptide (22 nmol/min per mg and 110 μ M respectively). Truncation of the peptide by four amino acids from the carboxyl terminal side did not have any effect on V_{max} whereas apparent K_m increased by 2-fold. Truncation of the peptide from the amino terminal side by five and six amino acids resulted in a decrease in K_m and V_{max} values. For both peptides, A-S-V-A and G-S-V-Y appeared to be the shortest phosphopeptides that could be dephosphorylated by PP2Ac. V_{max} values observed were 0.7 nmol/min per mg and 0.6 nmol/min per mg respectively. The two investigated phosphopeptides and their analogs showed apparent K_m values higher than the values obtained with their phosphoprotein substrates (168).

A series of synthetic peptides phosphorylated by the catalytic subunit of PKA were tested as substrates for PP2A and PP2C holoenzymes. Both enzymes showed a striking preference for the peptide R-R-A-I-V-A derived from the phosphorylation site sequence of pyruvate kinase, that is related to Kemptide, over R-R-A-S-V-A. The observed V_{max} values for PP2A dephosphorylation of the above two peptides were 11.7 pmol/min per ml and

316 pmol per min-ml respectively. The V_{max} values for the peptides' dephosphorylation by PP2C were 3.4 pmol/min per ml and 98.3 pmol/min per ml respectively. The strong preference for phosphothreonine was also observed with the synthetic peptide (R-R-S-I-V-A) which was phosphorylated on both the serine and threonine residue by PKA. The same were obtained when mixed casein phosphorylated on both serine and threonine residues by casein kinase 2. In addition, the PKA-phosphorylated synthetic peptides R-R-A-S-V-A, R-R-L-S-I-S-T-E-S and R-R-L-S-S-L-R-A derived from phosphorylation sites of pyruvate kinase, α -subunit of phosphorylase kinase and ribosomal protein S6 respectively were very poorly dephosphorylated (less than 10%) by PP2C compared to their protein counterparts. PP2C was completely inactive in dephosphorylating the PKA phosphorylated R-R-R-R-P-I-P-A synthetic peptide derived from the phosphorylation site of inhibitor-1. Modification of these peptides by various truncations and amino acid substitutions did not show a clear trend in improving the rate of dephosphorylation by either PP2A or PP2C (169).

McNall and Fischer (170) reported that the ability of PP1-G isolated from rabbit skeletal muscle to dephosphorylate synthetic phosphopeptides varied considerably depending on the form of the enzyme. Phosphorylase a phosphorylated by phosphorylase kinase and type 2 regulatory subunit phosphorylated by the catalytic subunit of PKA were both dephosphorylated by PP1-G, PP1c and PP1c in the presence of Mn^{+2} . Similar observations were made when synthetic phosphopeptides corresponding to the phosphorylation sites were tested as substrates for the above mentioned forms of PP1. The V_{max} values observed for the synthetic phosphopeptide dephosphorylations

were comparable to their protein counterparts. However, when PP1-G was phosphorylated by glycogen synthase kinase 3, the enzyme dephosphorylated the protein substrates but not the phosphopeptides. Likewise, PP1c isolated after PP1-G phosphorylation by glycogen synthase kinase 3 was unable to dephosphorylate phosphopeptides. These results suggested that the primary sequence surrounding the phosphorylate site is not all that is necessary for recognition by the phosphatase (170).

Collectively, these studies were contradictory and do not establish a clear role for the use of synthetic peptides in the determination of phosphoprotein phosphatase substrate specificity. In addition, the assays adopted in the above studies were dependent on the quantitation of the inorganic phosphate liberated in the phosphoprotein phosphatase-catalyzed reaction by either extraction in isobutanol/toluene of the phosphomolybdic complex (169, 171, 172) or ion exchange chromatography on AG50W-X2 and liquid scintillation counting (170). Neither of these methods permits simultaneous analysis of inorganic phosphate and unreacted peptide, and the time required for these analytical approaches severely limits the number of samples which can be conveniently processed in an assay.

Purpose of this study

The diversity of the sequence in the variable region of protein kinase C allows the separation of this enzyme into several isoenzymes upon chromatography on hydroxyapatite column (143-145). The relative activity and the individual pattern of expression of protein kinase C isoenzymes is

dependent on the tissue and the cell type in which protein kinase C is present (19, 112, 113, 141). The protein kinase C isoenzymes obtained thus far from various tissues exhibit some differences in enzymatic properties (143-145). The effector requirement differs from one isoenzyme to another (16). In addition, the protein kinase C isoenzymes show different preference for substrate proteins depending on their intracellular location in specific tissue in specific cell types (143-145).

Hypothesis

Some differences in apparent effector requirements may be correlated with discrete elements in the primary sequence of the protein kinase substrates. For example, occurrence of an acidic residue in the primary sequence may attenuate the requirement for phosphatidyl serine in the enzyme reactivity. These studies propose to test this hypothesis by using two highly homologous synthetic peptides which are protein kinase C substrates.

A number of studies have been conducted to demonstrate regulatory properties of various serine/threonine phosphoprotein phosphatase subclasses using different phosphoproteins as substrates, such as phosphorylase kinase, phosphorylase *a*, histones, and EGF receptor (167). However, firm conclusions are not possible in many cases since their protein substrates are difficult to prepare in substantial and sufficient quantities for routine use. In addition, these proteins are typically phosphorylated at a low stoichiometry at several different residues in the polypeptide chain, which makes it very difficult to determine the substrate specificity of phosphoprotein phosphatases.

The few attempts which have been made to study the specificity determinants for phosphoprotein phosphatases by utilizing synthetic phosphopeptides as substrates have failed to provide new insights into the selectivity of these enzymes. None of these studies have succeeded in defining consistent trends in kinetic parameters which can be correlated with the primary structure. In addition, most of these studies have been carried out with peptides modified only by cAMP-dependent protein kinases. No studies have investigated dephosphorylation of peptides modified by protein kinase C. This is of considerable interest since several PKC-dependent substrates are modified at threonine as opposed to serine residues.

A second hypothesis to be pursued in this dissertation is that the relative kinetics of PP1c and PP2Ac dephosphorylation of protein kinase C substrates can be established using synthetic peptides.

In this study, two peptides derived from the phosphorylation site of myosin light chain by protein kinase C were utilized to determine the local primary sequence as well as the effector requirements for protein kinase C purified from murine lymphosarcoma (P1798) tumors. These peptides were designated MLC(3-13) (K-R-A-K-A-K-T-T-K-K-R-G) and MLC(5-13) (A-K-A-K-T-T-K-K-R-G) and they differ in that the two basic residues, lysine and arginine, on the amino terminal of MLC(3-13) were truncated in MLC(5-13). These peptides were selected on the basis of previous studies which demonstrated that these threonine-containing peptides are excellent and well-defined substrates for protein kinase C (148). In addition, MLC(3-13) which was modified by protein kinase C with higher V_{max} and lower K_m values than MLC(5-13) was utilized as a substrate for the catalytic subunits of PP1 and PP2A (PP1c and PP2Ac

respectively) purified from murine lymphosarcoma tumors. These studies were conducted in order to determine whether these enzymes recognize the dephosphorylation site that was modified by protein kinase C from the primary amino acid sequence or whether a higher-ordered structure of the phospho-substrate is needed in order for dephosphorylation by these two phosphatases to occur. In addition, the effects of various activators and inhibitors on the dephosphorylation of phospho MLC(3-13) by PP1c and PP2Ac were established.

To investigate the generality of this observation, a second peptide derived from the ribosomal protein S6 and containing serine residues phosphorylated by an S6/H4 kinase was investigated. The peptide designated S6-21 (A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S-E-S-S-Q-K) was shown to be modified on at least three serine residues by an S6/H4 kinase purified from human placenta (173, 174). Finally, a novel method developed for phosphoprotein phosphatase analysis based on the principles established by Glass *et al.* (183) assay for protein kinases is described.

CHAPTER II

MATERIALS AND METHODS

Materials

[γ - ^{32}P]ATP (4500 Ci/mmol), [1- ^{14}C]glucose 1-phosphate (271 mCi/mmol) and Universol scintillation fluid were purchased from ICN Biomedicals Inc. (Irvine, CA). The inhibitor 2 (I-2) was a generous gift of Dr. Shirish Shenolikar, Department of Pharmacology, Duke University Medical Center, NC. Gizzard light chains were kindly supplied by Dr. James T. Stull, Department of Physiology, University of Texas Southwestern Medical Center at Dallas, TX. Heparin Sepharose was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). DEAE-52 anion exchange resin and phosphocellulose paper P81 were obtained from Whatman (Hillsboro, OR). AG 1x8 (acetate form), AG50W-X2 (hydrogen-form) and hydroxyapatite resins were purchased from Bio-Rad (Richmond, CA). Microcystin-LR from *Microcystis aeruginosa* was purchased from Calbiochem (San Diego, CA). Okadaic acid was purchased from Gibco-BRL (Grand Island, NY). Phosphatidyl serine (bovine brain, sodium salt) and oleoyl-2-acetoyl-3-glycerol (OAG) were purchased from Avanti Polar Lipids Inc. (Pelham, AL). Benzamidine hydrochloride, leupeptin, dithioerythritol, PMSF, CM Sephadex, phenyl Sepharose, S-200, Sephadex G-10, bovine serum albumin (fraction V), trypsin (bovine pancreas, type XI), soybean trypsin inhibitor (type I-S), thermolysin

(protease type X from *Bacillus thermoproteolyticus rokko*), protamine sulfate (essentially histone free), heparin (from porcine intestinal mucosa, grade I-A), Triton-X-100, poly L-lysine (Mr 4,000-15,000), glycogen (from oyster, type II), and phosphorylase a (rabbit muscle, 23 units/mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO).

All solvents for peptide synthesis and purification were HPLC grade. Reagents for peptide synthesis were purchased from Bio-Search (San Rafael, CA). All other reagents were of commercial sources and of highest purity available.

Methods

Tissue Source: Murine lymphosarcomas (P1798/R, cortisol-resistant) were serially transplanted subcutaneously in six- to eight-week-old female BALB/C mice. Tumors (14-18 g) were surgically removed 18 to 21 days after implantation, rinsed in cold water, and then homogenized for 60 sec in 3 volumes of cold 10 mM Tris-Cl, pH 7.5, containing 5 mM EDTA, 5 mM benzamidine, 1 mM leupeptin, 0.5 mM dithioerythritol (DTE), and 0.2 mM PMSF (buffer A). All subsequent steps were performed at 4°C unless otherwise indicated.

Purification of PP1c and PP2Ac: The catalytic subunits of phosphoprotein phosphatase type 1 and 2A (PP1c and PP2Ac) were purified from murine lymphosarcoma tumors to apparent homogeneity as described by DeGuzman and Lee (175) with some modifications. The tumors were homogenized in cold buffer A using a Waring blender for 1 min at maximum

speed. The homogenate was then centrifuged at 3,300 x g for 30 min, and the supernatant was filtered through glass wool. The volume of the supernatant solution was measured and brought to 70% saturation with ammonium sulfate. A few crystals of solid sodium bicarbonate were added prior and during the addition of the ammonium sulfate to maintain the pH near 7.5. When the ammonium sulfate was dissolved, the extract was allowed to stand for 1 hr without stirring. The precipitate was then collected by centrifugation at 3300 x g for 30 min, resuspended in buffer A, and homogenized in a Waring blender for 20 sec. Five volumes of room temperature 95% ethanol were quickly added to the suspension, and immediately centrifuged at 3300 x g for 10 min. The precipitate was extracted by homogenization with buffer A for 20 sec and centrifuged at 10,000 x g for 20 min. The extract was then dialyzed against 2 l of 12.5 mM Tris-Cl, pH 7.5, containing 1.25 mM EDTA, 0.25 mM DTE, 5 mM benzamidine and 0.2 mM PMSF (buffer B). Then it was dialyzed against 2.5 mM Tris-Cl, pH 7.5, containing 2.5 mM EDTA, 0.25 mM DTE, 5 mM benzamidine and 0.2 mM PMSF (buffer C) for 2 hr and overnight against 2 l of buffer A.

The dialyzed enzyme solution was filtered through glass wool and loaded onto a DEAE-52 column (1.5 x 30 cm), equilibrated with buffer A, at a flow rate of 1.5 ml/min. The column was washed with buffer A containing 100 mM NaCl, and was then eluted with a linear gradient of 100 mM to 500 mM NaCl. Fractions (~2.5 ml) collected were assayed for phosphatase activity using [³²P] S6-21 peptide as a substrate. Two peaks of phosphatase activity eluting at near conductivities of 6 and 15 mmho were resolved. The two phosphatase peaks were separately pooled. The second peak, which contains

PP2Ac activity, was dialyzed against Buffer A containing 50% glycerol (v/v) and stored at -20°C until further purification. The first peak, which contains PP1c activity, was dialyzed overnight against buffer A containing 20% glycerol (v/v). The volume of the second peak was approximately twice as large as the first one.

The dialyzed enzyme fractions were loaded onto a heparin Sepharose column (0.9 x 7 cm) equilibrated with buffer A containing 20% glycerol (v/v). The column was washed with buffer A containing 100 mM NaCl and 20% glycerol (v/v), and then eluted with buffer A containing 500 mM NaCl and 20% glycerol (v/v). Fractions (~2.5 ml) were collected at a flow rate of 1 ml/min and assayed for phosphatase activity using [³²P] S6-21 peptide as a substrate, and the active fractions were pooled. The next step was determined according to the volume of the partially purified enzyme eluted from heparin-Sepharose column, which is dependent on the amount of starting tissue. When the volume obtained was more than 10 ml, the pooled fractions were concentrated to ~5 ml using an Amicon ultrafiltration cell with a YM5 membrane and then loaded on an S-200 column (2.5x90 cm) equilibrated with buffer A containing 300 mM NaCl and 20% (v/v) glycerol. If the volume of enzyme eluted from the heparin Sepharose column was less than 5 ml, the pooled fractions were concentrated to ~200 µl using an Amicon cell with a YM5 membrane and then loaded onto an FPLC Superose 12 gel filtration column equilibrated with buffer A containing 300 mM NaCl and 10% (v/v) glycerol. In either case, the columns were eluted with the equilibration buffer at a flow rate of 0.5 ml/min and fractions (0.5 or 3.8 ml) were collected. Protein-containing fractions were determined by Bio-Rad dot assay. Aliquots (30 µl) were added to 8 µl of Bio-

Rad dye and mixed together. After two minutes of incubation, fractions containing protein developed a blue colored complex. These fractions were assayed for phosphatase activity using [^{32}P] S6-21 peptide as a substrate. Active fractions were pooled and stored at -80°C in 200 μl aliquots.

The second peak obtained from the DEAE-52 chromatography was diluted 1:1 with buffer A and passed through a heparin-Sepharose column (0.9x7 cm) equilibrated in buffer A containing 20% glycerol (v/v), in order to remove any PP1c contamination. The eluate was then concentrated and chromatographed on either S-200 or Superose 12 FPLC column as described above. Active fractions (~ 3.8 ml or 0.5 ml, depending on the column used) were pooled and stored at -80°C in 200 μl aliquots. Purity was determined by silver staining of 12.5% SDS-PAGE. The specific activities for PP1c and PP2Ac were $24 \mu\text{mol}/\text{min}\cdot\text{mg}^{-1}$ and $0.77 \mu\text{mol}/\text{min}\cdot\text{mg}^{-1}$ respectively with phosphoMLC(3-13) as a substrate.

PP1c and PP2Ac were also purified from rabbit skeletal muscle essentially as described above.

Purification of Protein Kinase C: Protein kinase C was purified from murine lymphosarcoma P1798 tumors as previously described (148). Tumors (4-8 g) were surgically removed from 8- to 10-week-old female BALB/C mice and homogenized in 3 volumes of 10 mM Tris-Cl, pH 7.5, containing 2 mM EDTA, 2 mM EGTA, 30 mM β -mercaptoethanol (BME) and 0.1 mM PMSF (buffer D). All subsequent procedures were conducted at 4°C unless otherwise indicated. The homogenate was centrifuged at $13,300 \times g$ for 15 min and the supernatant was filtered through glass wool. The supernatant was

centrifuged at 170,000 x g for 60 min. The supernatant was diluted with cold deionized water until the conductivity was less than 2 mmho. The sample was then batch loaded onto 45-50 ml of DEAE-52 anion exchange resin equilibrated with buffer D. The DEAE-52 was poured into a column. The column was washed with buffer D and then eluted with a linear gradient of 0-500 mM KCl. Fractions (~2.5 ml) collected were assayed for protein kinase C activity using protamine as a substrate in the absence of effectors. The active fractions near conductivity of 8 mmho were pooled, concentrated by precipitation with 60% ammonium sulfate, and dialyzed against buffer D for 1 hr and then with buffer E (10 mM Tris-Cl, pH 7.5, containing 2 mM EDTA, 0.5 mM EGTA, 30 mM BME and 0.1 mM PMSF) for 1 hr. The sample was then dialyzed against buffer F (10 mM potassium phosphate, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA and 10 mM β -mercaptoethanol) until the conductivity was less than 5 mmho. The sample was then mixed with 6-8 ml hydroxyapatite equilibrated with buffer F. The resin was batch washed with buffer F containing 400 mM KCl and then with buffer F. The hydroxyapatite was poured into a column and further washed with buffer F until the effluent had a conductivity of less than 4 mmho. The protein kinase C activity was eluted with buffer F containing 300 mM potassium phosphate, pH 7.5 (buffer H). The active fractions (~2.5 ml) near conductivity of 30 mmho were pooled and dialyzed overnight against 1 l of buffer D. The dialyzed sample was loaded on CM sephadex (~3 ml) equilibrated with buffer D. The column was washed with buffer D and the flow through (12-15 ml) was collected. KCl was added to the sample so that the final concentration would be 1.5 M. The sample was loaded onto a phenyl Sepharose column (~4 ml) equilibrated in buffer I (10 mM Tris-

Cl, pH 7.5, containing 1 mM EDTA, 30 mM β -mercaptoethanol and 1.5 M KCl). The column was then eluted with a linear gradient of 1.5-0 M KCl. Fractions (~2.5 ml) collected were assayed for protein kinase C activity using MLC(3-13) peptide as the substrate in the presence and absence of effectors. The most active fractions near conductivity of 20 mmho were pooled, dialyzed against buffer A containing 10% glycerol (v/v), and stored at -80°C in 100 μ l aliquots.

Peptide Synthesis: S6-21 (A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S-E-S-S-Q-K) is a synthetic peptide derived from the carboxyl terminal sequence of the ribosomal protein S6. MLC(3-13) (K-R-A-K-A-K-T-T-K-K-R-G) and MLC(5-13) (A-K-A-K-T-T-K-K-R-G) are synthetic peptides derived from protein kinase C phosphorylation site(s) at the amino terminus of smooth muscle myosin light chain. Both peptides were synthesized as the COOH-terminal acid from t-BOC amino acids using a SAM II solid phase peptide synthesizer (Milligen Biosearch) as previously described (176, 177). After the synthesis, the peptides were cleaved and deprotected using distilled anhydrous hydrofluoric acid. The cleaved peptides were concentrated, desalted, and purified using a semipreparative Bio-Rad C-18 reverse phase HPLC column (1.0 x 25 cm). The peptides were purified and eluted with a linear gradient (0-60%) of acetonitrile containing 0.1% trifluoroacetic acid. The gradient time was 44 min with a flow rate of 2 ml/min. The peptide was eluted and detected spectrophotometrically at a wavelength of 220 nm. After the major peak was pooled, sequences were confirmed by sequence analysis on an Applied Biosystems 470A gas phase sequencer equipped with a model 120A on-line phenylthiohydantoin analyzer.

After the sequence confirmation, the peptides were lyophilized and stored dry at -20°C until used. Before use, peptides were neutralized to pH 7.0 by the addition of 0.01 M NaOH.

Phosphorylation of S6-21: The S6-21 peptide was phosphorylated by an activated and trypsinized S6/H4 kinase isolated from human placenta. S6/H4 kinase (10 µg, ~100 µl) was incubated for 4 min at 30°C with 50 µl of 50 mM MES, pH 6.8, containing 200 µg bovine serum albumin and 0.6 µg trypsin. The trypsinization reaction was stopped by the addition of 1.2 µg soybean trypsin inhibitor in 10 mM Tris-Cl, pH 7.6 (50 µl). The trypsinized S6/H4 kinase was incubated for 10 min at 30°C with 50 µl of 1 mM [γ -³²P]ATP (550-950 dpm/pmol), 50 µl of 10 mM MgCl₂ and 100 µl of 50 mM Tris-Cl, pH 7.5. S6-21 (750 µg) was added to the incubation mixture. The final reaction mixture (500 µl) was incubated for 2 hr at 30°C. The phosphorylation reaction was stopped by the addition of acetic acid to 30% final concentration and the phosphopeptide was separated from the unreacted [γ -³²P]ATP by anion exchange chromatography on AG 1x8 (acetate form, ~2 ml) equilibrated with 15% acetic acid (166). The product was lyophilized and an aqueous stock solution was stored at -80°C. The stoichiometry of the phosphate incorporation into peptide was up to 2 mol of phosphate transferred/mol of peptide.

Preparation of mixed micelles: Mixed PS and OAG micelles were prepared to a final volume of 25 µl or its multiples essentially as previously described (178, 179). Only glass tubes and syringes were used for the preparation of mixed micelles. Stock solutions of PS and OAG were in chloroform. To prepare 25 µl of mixed micelles, PS (109 nmol) and OAG

(22 nmol), unless otherwise indicated, were pipetted to a glass test tube. The chloroform was evaporated with nitrogen gas. The residue was then dissolved in 3% (w/v) Triton-X-100 in water (25 μ l), so that the final concentrations of PS and OAG with respect to Triton-X-100 solution were 10 mol% and 2 mol% respectively. The mixture was allowed to sit for 5 min at room temperature and then vortexed vigorously at high speed for 2 min. Within 30 min, the mixed micelles were used for protein kinase C assays (5 μ l mixed micelles/50 μ l reaction mixture).

Phosphorylation of MLC(3-13): The MLC(3-13) was phosphorylated with 0.1 mM [γ - 32 P]ATP (~500-600 dpm/pmol), using 20 mM Tris-Cl, pH 7.5, containing 10 mM MgCl₂, 0.5 mM CaCl₂, and 50 μ l of mixed micelles (10 mol% PS and 2 mol% OAG) in a total volume of 500 μ l unless otherwise indicated. MLC(3-13) (1.5 nmoles) along with the effectors were incubated with protein kinase C (0.6 μ g) for 1 hr at 30°C. The phosphorylation reaction was stopped by the addition of acetic acid to 30% final concentration and the phosphopeptide was separated from the unreacted [γ - 32 P]ATP by AG 1x8 essentially as described above. The stoichiometry of the phosphate incorporation into peptide was up to 2 mol of phosphate transferred/mol of peptide.

Phosphorylation of gizzard light chains: Gizzard light chains were phosphorylated with 0.1 mM [γ - 32 P]ATP (~270 cpm/pmol), using 20 mM Tris-Cl, pH 7.5, containing 10 mM MgCl₂, 0.5 mM CaCl₂, and 50 μ l of mixed micelles (10 mol% PS and 2 mol% OAG) in a total volume of 500 μ l. Gizzard light chains (725 pmol) along with the effectors were incubated with protein

kinase C (~0.6 μ g) for 1 hr at 30°C. The phosphorylation reaction was stopped by the addition of acetic acid to 30% final concentration and the phosphoprotein was separated from the unreacted [γ - 32 P]ATP by AG 1x8 essentially as described above. The stoichiometry of phosphate incorporation into the light chains was ~0.16 mol of phosphate transferred/mol of gizzard light chains.

Analysis of phosphopeptides: PhosphoS6-21 was dephosphorylated by either PP1c or PP2Ac. The dephosphorylation reaction was terminated by diluting the reaction mixture with 0.1% (v/v) trifluoroacetic acid in water for HPLC analysis or by incubating the mixture in a boiling water bath for 1 min for digestion by thermolysin (180). The mixture was cooled to room temperature and incubated with thermolysin solution (250 μ g thermolysin in 500 μ l Tris-Cl, pH 8.0, containing 2-3 μ l of 1 M NaOH) (1.5% w/w) for 90 min. Then thermolysin solution (1.5% w/w) was added once more for another 90 min. The reaction mixture was then acidified by the addition of 950 μ l of 15% acetic acid and chromatographed on AG 1x8 (acetate form, ~2 ml) essentially as described above to remove inorganic phosphate and lyophilized prior to HPLC analysis. The lyophilized material was reconstituted in 0.1% trifluoroacetic acid in water. Reverse phase HPLC was conducted on a C18 octadecyl bonded phase 5 μ M column (Gilson) equilibrated with 0.1% trifluoroacetic acid. The column was eluted with a linear gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid. Fractions were collected and 20 μ l aliquots were spotted on P81 paper squares (1x1 cm) and quantitated by liquid scintillation counting in toluene-based fluor. HPLC analysis was also conducted with thermolysin-digested phospho S6-21, undigested phosphoS6-

21, dephosphorylated undigested S6-21, and phosphorylated undigested MLC(3-13).

Sequence analysis of the proteolytic products of phospho S6-21 was conducted essentially as described previously for the parent peptide.

Amino acid composition analysis of thermolysin-digested peptide: The amino acid composition of the major proteolytic peptide resulting from thermolysin digestion of phosphoS6-21 was determined. Analysis was conducted by the Biopolymer Analysis Laboratory of TCOM according to the method of Westall and Hesser for peptides less than 30 residues (181).

Analysis was performed with a Varian 5560 Liquid Chromatograph interfaced with a Pickering flow conditioner and Eldex A-30 pump for post column derivatization, a Gilson 121 Fluorometer as detector, and a Varian DS 601 Data System. Amino acids were eluted from a Pierce High Speed AA511 column using a gradient based on the Pierce Buffelute Hydrolyzate Buffer system. Analytical conditions were 63°C, eluent flow of 0.5 ml/min, and reagent flow of 0.2 ml/min. Detection was by post column derivatization with orthophthaldehyde (182).

Protein kinase C assay: Protein kinase C assays were conducted essentially as previously described (183). Each reaction mixture (50 μ l) contained 20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM [γ - ^{32}P]ATP (30-200 dpm/pmol), lipid effectors, substrate (10 μ g of either MLC(3-13) or MLC(5-13), and 5 μ l of ~6.0 μ g/ml of enzyme unless otherwise indicated. When lipid effectors were used in the form of micelles, 5 μ l of 10 mol% PS and 2 mol% mixed micelles (see above) were used. Otherwise,

where indicated, PS and OAG were 1 mg/ml and 3.5 µg/ml respectively. Stock solutions of PS and OAG were in chloroform. The chloroform was evaporated with nitrogen gas, and the residue was redissolved in water by vigorous vortexing before use in the assay. Incubation was for 10 min at 30°C. The reaction was terminated by spotting a 35 µl aliquot onto a P81 paper (1 x 2 cm) and transferring the paper immediately to cold 0.6% phosphoric acid solution (500 ml) washed for 12 min. The second and third washes were in cold 0.3% phosphoric acid solution for 12 min. The fourth and fifth washes were in room temperature 0.3% phosphoric acid for 6 min. The final wash was in acetone at room temperature for 4 min. The acetone was decanted and the papers were dried with a blow dryer. The radioactivity was determined by liquid scintillation counting in a toluene-based fluor.

PhosphoS6-21 phosphatase assay: Peptide dephosphorylation assays were conducted in 50 µl containing 20 mM Tris-Cl containing 0.1 mM PMSF and 0.1 mM DTE, pH 7.5, 7 µM [³²P] S6-21 (50-750 dpm/pmol peptide), with either PP1c or PP2Ac, activators, and inhibitors as described in the individual experiments. The reaction mixture was incubated at 30°C for 10 min unless otherwise indicated, after which 35 µl was pipetted onto a 2 cm diameter P81 paper circle and each paper was immediately immersed in 1 ml 50 mM NaH₂PO₄, pH 6.0, in a 20 ml glass liquid scintillation vial. After all assays were terminated, the vial contents were mixed and the P81 papers were partially removed from the vial and allowed to drain on the inner circumference of the vial for approximately 1 min. The P81 paper was removed from the vial and [³²P] phosphate in the NaH₂PO₄ was quantitated by liquid scintillation

counting in 7.5 ml Universol. P81 papers were either discarded or transferred to a wire mesh screen (182) and dried with a warm air stream. [^{32}P] S6-21 remaining on the dried paper was quantitated by liquid scintillation counting in a toluene-based fluor.

PhosphoMLC(3-13) phosphatase assay: MLC(3-13)

dephosphorylation assays were conducted in 50 μl containing either PP1c or PP2Ac, 5.8 μM [^{32}P] MLC(3-13) (500-700 dpm/pmol peptide), and 20 mM Tris-Cl, pH 7.5, containing 0.1 mM PMSF and 0.1 mM DTE. The reaction mixture was incubated at 30°C for 10 min unless otherwise indicated. The reaction was stopped and the radioactive inorganic phosphate was quantitated essentially as described for the dephosphorylation of [^{32}P] S6-21.

Gizzard phospholight chain phosphatase assay: Gizzard light

chain dephosphorylation assays were conducted in 50 μl containing either PP1c or PP2Ac, 7.25 μM [^{32}P] gizzard light chains (200-260 cpm/pmol protein), and 20 mM Tris-Cl, pH 7.5, containing 0.1 mM PMSF and 0.1 mM DTE. The reaction mixture was incubated at 30°C for the indicated time period. The reaction was stopped and the radioactive inorganic phosphate was quantitated essentially as described for the dephosphorylation of [^{32}P] S6-21.

Phosphorylase phosphatase assay: Glycogen phosphorylase a

activity was measured in the direction of glycogen synthesis using [1- ^{14}C]glucose 1-phosphate (2.5 $\mu\text{Ci}/\text{mmol}$). The assays were conducted in 50 mM MES buffer, pH 6.8, according to the procedure of Gilboe *et al.* (184). Either PP1c or PP2Ac were incubated with phosphorylase a for 10 min in a total

volume of 25 μ l. The dephosphorylation reactions were stopped by the addition of microcystin-LR to a final concentration of 2 μ M. The glycogen synthesis reaction was allowed to proceed by the addition of 0.6 mg glycogen (type III, oyster muscle) and 60 mM [$1\text{-}^{14}\text{C}$]glucose 1-phosphate with or without 1.4 mM AMP in a total volume of 70 μ l. Reactions were allowed to proceed for 10 min after which 42 μ l aliquots were spotted onto 2 cm² P81 papers and immediately immersed in 66% cold ethanol and washed for 10 min. The second wash was conducted at room temperature with 66% ethanol for another 10 min. Finally, papers were washed with acetone for 2 min and dried with a warm air stream. [^{14}C]Glycogen on the dried papers was quantitated by liquid scintillation counting.

Other: The S6/H4 kinase was purified from human placenta as previously described (173,174). Protein concentrations were determined by the dye binding method of Bradford (185) using bovine serum albumin as the standard. In most cases experiments were conducted in duplicates.

CHAPTER III

RESULTS

Peptide Synthesis: Synthetic peptides have been utilized extensively to facilitate the determination and analysis of the substrate specificity of several protein kinases (151-165). The reason behind utilizing synthetic peptides is the observation that the substrate specificity of several protein kinases has been shown to be determined by the primary amino acid sequence in the vicinity of the phosphorylation site in the substrate. In this study, three synthetic peptides have been utilized to investigate the reversible phosphorylation by protein kinase C and the catalytic subunits of phosphoprotein phosphatases 1 and 2A (PP1c and PP2Ac respectively). Highly homologous peptides MLC(3-13) (K-R-A-K-A-K-T-T-K-K-R-G) and MLC(5-13) (A-K-A-K-T-T-K-K-R-G) corresponding to the amino terminal amino acid sequence of smooth muscle myosin light chain containing protein kinase C phosphorylation site(s) were synthesized. In addition, an S6-21 peptide (A-K-R-R-R-L-S-S-L-R-A-S-T-S-K-S-E-S-S-Q-K) corresponding to the carboxy terminus of the ribosomal protein S6 was also synthesized. Yields of pure peptides ranged from 40-60%. To confirm the predicted amino acid sequences, all purified peptides were analyzed by gas phase sequencing. With all three peptides, the predicted sequences were confirmed.

1. Protein kinase C

Rationale: Although a great deal of work has been described in regard to protein kinase C responses to extracellular stimuli, little has been done regarding the substrate specificity of this enzyme. It has been reported that the substrate specificity determinants of protein kinases are dependent on the local primary amino acid sequence in the vicinity of the phosphorylation site on the protein substrate (151-154). Basic amino acids such as lysine and/or arginine are primary determinants for many protein kinase specificities such as cyclic AMP - dependent protein kinase and protein kinase C (146-150). In addition, convincing evidence has been presented regarding the role of substrate in influencing the effector requirements of protein kinase C (127, 128). For example, substrates like protamine do not require effectors in order to be modified by protein kinase C, whereas myelin basic protein requires the presence of phospholipid only. Substrates such as histone and myosin light chain require the presence of Ca^{+2} , phospholipid and diacylglycerol. In this report, the effector requirement for protein kinase C activation using two highly homologous synthetic peptides MLC(3-13) (K-R-A-K-A-K-T-T-K-K-R) and MLC(5-13) (A-K-A-K-T-T-K-K-R) as substrates is presented. Knowledge of the role of substrate in defining the effector requirements for protein kinase C activation is important for understanding the *in vivo* regulation of the enzyme and defining its substrates.

Effector requirements: In the absence of calcium and lipid effectors, phosphotransferase activity of protein kinase C with MLC(3-13) and MLC(5-13)

were 0.9 pmol phosphate incorporated/min and 0.7 pmol phosphate incorporated/min for MLC(3-13) and MLC(5-13) respectively (Table I). The addition of CaCl_2 or water-dispersed phosphatidyl serine or diacylglycerol alone did not significantly increase protein kinase C activity with either of the peptides. With both peptides, the addition of CaCl_2 and PS, or CaCl_2 and OAG together resulted in a moderate increase in the enzyme activity. The addition of phosphatidyl serine and OAG resulted in 17- and 16-fold increases in protein kinase C activity with MLC(3-13) and MLC(5-13) respectively. However, when 50 μM CaCl_2 , 5 μg phosphatidyl serine, and 0.175 μg diacylglycerol were added together to the reaction mixture, a 35- and 32-fold increase in phosphate transfer was observed with MLC(3-13) and MLC(5-13) respectively.

Studies on the *in vitro* regulation of protein kinase C activity by diacylglycerol, phospholipids, and calcium have generally utilized physically undefined, nonhomogeneous sonic lipid dispersions (186-191) as demonstrated above. Mixed micelles of Triton-X-100, containing phosphatidyl serine and diacylglycerol have been demonstrated to provide a physically defined, homogeneous system for studies on the stoichiometry and specificity of protein kinase C activity by lipids (178-179).

To investigate further the effector requirements for protein kinase C activation with the peptide substrates, a micellar assay using the same synthetic peptides utilized as substrates in the standard assay (reported above) was employed. The addition of CaCl_2 , phosphatidyl serine and OAG alone did not significantly increase protein kinase C activity with either of the peptides (Table II). Unlike the standard assay, with both peptides, the combination of phosphatidyl serine and diacylglycerol had little effect on protein kinase C

activity. However, in the presence of 50 μM CaCl_2 , 10 mol% phosphatidyl serine and 2 mol% diacylglycerol mixed micelles, a synergistic effect on protein kinase C activity was observed. Protein kinase C activity with MLC(3-13) and MLC(5-13) increased by 34- and 30-fold respectively.

As shown in Figure 1, under saturating conditions of phosphatidyl serine and diacylglycerol, protein kinase C was stimulated to maximum activity in the presence of 25 μM CaCl_2 when assayed by the standard sonic dispersed lipid procedure. On the other hand, 50 μM CaCl_2 was required for maximal protein kinase C activity in the micellar assay when 10 mol% phosphatidyl serine and 2 mol% OAG mixed micelles were used (Fig. 1). In addition, protein kinase C activity was approximately 30% higher with micellar assay than that with the standard assay (Fig. 1).

Phosphatidyl serine and diacylglycerol dependence of protein kinase C activation: A major difficulty in investigating the role of lipid in protein kinase C activation is the physical status of the lipid component. As reported above, phosphatidyl serine and OAG were shown to activate protein kinase C whether they were in the form of dispersed vesicles or mixed micelles. However, their protein kinase C activation showed significant differences in the two systems. The observation that protein kinase C activity is higher when assayed with mixed lipid micelles provided a well-defined system to investigate the stoichiometry and specificity of protein kinase C activation. The separate requirements for phosphatidyl serine and OAG for protein kinase C activation were investigated using the micellar assay system with both peptide substrates MLC(3-13) and MLC(5-13).

The dependence of protein kinase C activation on OAG under saturating conditions of phosphatidyl serine in the presence of 50 μM CaCl_2 was investigated. With MLC(3-13), the observed K_a for OAG was 0.57 ± 0.05 mol% whereas that for MLC(5-13) was 0.41 ± 0.01 mol% (Fig. 2).

The phosphatidyl serine dependence of protein kinase C under saturating conditions of OAG in the presence of 50 μM CaCl_2 was investigated (Fig. 3). With both peptides, 10 mol% phosphatidyl serine in mixed micelles was required before full protein kinase C activation became evident (Fig. 3). The PS dependence of protein kinase C activation was cooperative. At concentrations higher than 10 mol% phosphatidyl serine, a slight decrease in phosphate transfer was observed (Fig. 3), suggesting that phospholipid concentrations beyond saturation may involve unspecific binding to another domain on the protein kinase C molecule, resulting in its inhibition. In addition, Hill coefficients of 1.8 and 1.9 were calculated from these data for MLC(3-13) and MLC(5-13) respectively (Fig. 4A, B). These results suggest that with both synthetic peptides two molecules of phosphatidyl serine in mixed micelles may be sufficient in order for protein kinase C activation to occur.

In order to gain more insight about the lipid-dependent activation of protein kinase C activity, the order of binding of phosphatidyl serine and diacylglycerol to protein kinase C was examined. In the presence of 50 μM CaCl_2 , the addition of phosphatidyl serine to the reaction mixture 5 min after the addition of OAG did not have any effect on the amount of phosphate incorporated into MLC(3-13) (Table III). Likewise, the addition of OAG 5 min after the addition of phosphatidyl serine to the reaction mixture resulted in similar protein kinase C activity as the control reaction to which both effectors

were added at the same time. With MLC(5-13) as a substrate, similar results were obtained (Fig. 5). These data suggest that binding of phosphatidyl serine and OAG to protein kinase C is random and the binding of one effector to its binding domain on the enzyme does not block the binding of the other effector.

Collectively, the above data support the hypothesis that basic residues on the amino terminal side of the phosphorylation site are important specificity determinants for protein kinase C. Both of the highly homologous synthetic peptides MLC(3-13) and MLC(5-13) were phosphorylated by protein kinase C. With MLC(3-13), protein kinase C activity was 43 pmol/min compared to 27 pmol/min with MLC(5-13). This confirms the hypothesis that basic amino acid residues, lysine and arginine, located on the amino terminus of MLC(3-13) which were truncated in MLC(5-13) did indeed serve as important specificity determinant for protein kinase C. Due the presence of these basic amino acid residues in proximity of the phosphorylation site in MLC(3-13), the peptide was modified approximately twice as fast as MLC(5-13) which lacks those residues in its primary structure. The K_a values obtained for OAG with both MLC(3-13) and MLC(5-13) were comparable (0.57 and 0.41 respectively). Results from this laboratory have shown that substituting the amino terminal residues lysine and arginine in MLC(3-13) with two alanine residues did not improve the kinetic constants (Masaracchia, R., personal communication). These data suggest that the presence of basic residues such as lysine or arginine in the primary structure of synthetic peptide substrates may not have an effect on the requirement of OAG for the enzyme activation. However, the generality of this observation is yet to be investigated. Furthermore, protein kinase C activity was 20-30% higher with mixed micellar assay than that with sonic dispersed lipid

assay (standard assay). A major difference, however, is that in the absence of calcium, the presence of sonicated phosphatidyl serine and diacylglycerol significantly increased protein kinase C activity with the standard assay compared to the phosphatidyl serine-OAG mixed micelles which had little effect on the enzyme activity. In both assay procedures, and with both peptide substrates, CaCl_2 alone had little effect on protein kinase C activity. However, a synergistic effect of CaCl_2 , phosphatidyl serine and diacylglycerol was observed. The order of addition of OAG and phosphatidyl serine to protein kinase C reaction mixture did not affect the enzyme activity, suggesting that binding of one effector to protein kinase C is totally independent of binding of the other effector to the enzyme.

Finally, the above data demonstrate that protein kinase C purified from murine lymphosarcoma tumors meets the criteria of PKC- α in terms of its dependency on calcium, phosphatidyl serine and OAG. All three effectors are required for maximum activity of the enzyme. These data are in strong agreement with unpublished data from our lab demonstrating that protein kinase C isolated from murine lymphosarcoma tumors cross reacted with anti-PKC- α antibodies (Masaracchia, personal communication).

2. Phosphoprotein phosphatases

Rationale: The reversible phosphorylation of enzymes and other target proteins is an important mechanism for the regulation of intracellular processes. The specificity of this mechanism depends on the cellular protein kinases and phosphoprotein phosphatases which catalyze the phosphorylation and

dephosphorylation reactions respectively. The regulation and specificity determinants of protein kinases has been intensively investigated. More recent interest has focused on the phosphoprotein phosphatases which catalyze the physiological reversal of the phosphorylation reactions.

Despite the substantial progress in elucidating the physical and regulatory properties of phosphoprotein phosphatases, little is known about the substrate specificities of these enzymes. This is, in part, due to the lack of simple, well-defined substrates for these enzymes, for most of the protein substrates are difficult to prepare in sufficient quantities and are typically phosphorylated at a low stoichiometry at several different residues in the polypeptide chain. Another reason for the little information on the specificity determinants of phosphoprotein phosphatases is the lack of a simple and reliable assay procedure.

The dephosphorylation of a well-defined protein kinase C synthetic peptide, MLC(3-13), by the catalytic subunits of phosphoprotein phosphatase 1 and 2A (PP1c and PP2Ac respectively) is described. The dephosphorylation of an S6 peptide modified on multiple sites by an S6/H4 kinase is also described. Knowledge of dephosphorylation of peptides modified by two different kinases establishes the relative kinetics of PP1c and PP2Ac dephosphorylation. This should determine whether these enzymes recognize dephosphorylation sites from the primary amino acid sequences of substrates or a higher ordered structure is needed in order for dephosphorylation by these two phosphatases to occur.

Peptide dephosphorylation assay: A few preliminary attempts have been made to apply to the synthetic peptide strategy to the determination of phosphatase substrate specificity. These attempts were contradictory and failed to provide new insights into the specificity determinants of phosphoprotein phosphatases. This is, in part, due to the lack of simple and reliable assay procedure for these enzymes. In these studies, inorganic phosphate liberated in the phosphoprotein phosphatase-catalyzed reaction was quantitated by extraction in isobutanol/toluene of the phosphomolybdic complex (171, 172) or ion exchange chromatography on AG50W-X2 and liquid scintillation counting (170). Neither of these methods permit simultaneous analysis of inorganic phosphate and unreacted peptide, and the time required for these analytical approaches severely limits the number of samples which can be conveniently processed in an assay.

To facilitate the analyses of phosphopeptide dephosphorylation by PP1c and PP2Ac, it was necessary to develop a simple and reliable assay procedure for phosphoprotein phosphatases. Initially, the assay procedure was developed with PP1c and phosphoS6-21. Later it was applied to PP2Ac and phospho MLC(3-13).

Dephosphorylation of [^{32}P]S6-21 by PP1c was determined by the phosphocellulose paper method described under Materials and Methods and by the ion exchange chromatography method (170). With both methods, the release of phosphate was linear up to 12 min (Fig. 5). The activity measured by the ion exchange method was approximately 10% less than that measured by the phosphocellulose method at all time points except 2 min, which

demonstrates that the sensitivity of the paper method assay is comparable if not higher than the ion exchange method.

In order to determine the relationship between phosphopeptide and background in the assay, varying amounts of [^{32}P]S6-21 in absence of enzyme were pipetted onto phosphocellulose circles and the papers were washed in NaH_2PO_4 as described in Materials and Methods. Radioactivity in the phosphoric acid wash and on the phosphocellulose papers was determined by liquid scintillation counting. Results show that the assay background increased as the amount of radioactivity in the assay was increased, but the increase was not strictly proportional to the total amount of [^{32}P]S6-21 in the assay (Fig. 6). As the amount of peptide was increased from 25 to 350 pmol/paper, the proportion of radioactivity appearing as nonspecific background declined from 5.0% to 2.2% of the total dpm.

To test the effect of washing time on assay background, [^{32}P]S6-21 was pipetted onto P81 circles and the papers were allowed to stand in the NaH_2PO_4 wash for variable lengths of time from 1 min to 20 min. Results are shown in Figure 7. Washing the papers for less than 5 min resulted in variable background levels. After 5 min washing, the background in the wash was 2.8% of the total radioactivity applied to the phosphocellulose papers. This level of background activity did not change for washing times up to 20 min.

The binding of [^{32}P]S6-21 to phosphocellulose was determined in the presence of several buffers which are commonly used for phosphoprotein phosphatase assays. Both the background radioactivity in the wash and the phosphopeptide bound to the phosphocellulose paper were determined in order to ensure that the buffers had no effect on quantitation of radioactivity in

the wash or on the filter. Results are shown in Table IV. With 140 pmol/paper, the highest assay background count was 4.8% when 20 mM MES, pH 6.8, was used. Using Tris (20 mM), pH 7.5, resulted in the lowest assay background (2.9%). The presence of HEPES, pH 7.0, or β -glycerophosphate, pH 6.8, resulted in a background of 4.1%. With 350 pmol/paper, lowest assay background detected was 2.6 when either HEPES, pH 7.0, or Imidazole, pH 6.5, were used. Highest assay background was observed with 20 mM Tris (3.9%) (Table IV). In general, none of the buffers tested significantly affected the amount of background radioactivity that was detected in the phosphoric acid wash. In addition, none of the buffers influenced the recovery of phosphopeptide on the phosphocellulose filters.

The studies of the effects of buffer composition on assay backgrounds and binding of [32 P]S6-21 to the phosphocellulose filters were extended to include the effects of several commonly used phosphoprotein phosphatase effectors. The presence of 10 mM and 50 mM MgCl_2 did not have any effect on the phosphatase assay background (2.9% of total radioactivity). The assay backgrounds in the presence of 50 mM MnCl_2 and 50 mM of the non-specific phosphatase inhibitor NaF were 3.7 and 3.1% respectively (Table V). Similarly, the addition of the widely used tyrosine phosphatase inhibitor sodium *orthovanadate* (50 mM) and the non-specific phosphatase inhibitor sodium phosphate (100 mM) resulted in background activity of 3.2 and 2.9% of the total radioactivity respectively. Microcystin-LR, a specific inhibitor of PP1 and PP2A, did not have any effect on the phosphatase assay background (2.8% of total radioactivity applied to the phosphocellulose paper) (Table V). Generally, none

of these effectors increased the assay background or decreased the recovery of phosphopeptide on the phosphocellulose papers.

HPLC analysis of peptide dephosphorylation: In order to further examine peptide dephosphorylation assay by phosphocellulose paper method, HPLC analysis of peptide dephosphorylation was conducted. The molar ratio of phosphate incorporated into PKC-phosphorylated MLC(3-13) is approximately 2 to 1. This indicates that the two adjacent threonine residues which represent the only potential phosphorylation sites on the peptide were indeed phosphorylated. PhosphoMLC(3-13) was eluted with a linear gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid from reverse phase C18 column. The phosphopeptide was eluted in fractions 44-46 (32% acetonitrile) (Fig. 8). The volume of fractions collected was 0.5 ml each. When the phosphopeptide (0.4 nmoles) was incubated with PP1c (1 ng) for 6 min, and then loaded on a C18 column, two radioactive peaks appeared on the elution profile (Fig. 9). The first and second radioactive peaks eluted in fractions 7-9 and 46-48 respectively. The first peak fractions were pooled and the absorbance at 220 nm did not indicate the presence of peptide material. In addition, 20 μ l of the same pool as spotted on P81 paper, washed with NaH_2PO_4 as described for the P81 paper assay, and the radioactivity was quantitated on a scintillation counter. Radioactivity detected on the P81 paper was approximately 2.9% of that in the NaH_2PO_4 wash, demonstrating that the radioactive material did not bind to the phosphocellulose paper. This experiment establishes that the first radioactive peak contained radioactive inorganic phosphate liberated from the dephosphorylation reaction by PP1c. The second radioactive peak which

contained the partially dephosphorylated peptide eluted in fractions 46-48 (as reported above), two fractions after phosphoMLC(3-13) eluted (see above). This is due to partial loss of the phosphate content of the peptide after dephosphorylation with PP1c which resulted in an increase of the peptide's hydrophobicity and thus longer retention time on the column. Longer incubation of phosphoMLC(3-13) with the phosphatase results in larger radioactive inorganic phosphate peak and smaller radioactive peak which contains the partially dephosphorylated peptide. Figure 10 illustrates the elution profile of phosphoMLC(3-13) (0.3 nmoles) dephosphorylated for 20 min by PP2Ac (17 ng).

To investigate if phosphoMLC(3-13) is completely dephosphorylated by PP1c (1 ng), the peptide (0.3 nmoles) was incubated with PP1c and the dephosphorylation reaction was allowed to proceed for 60 min. At the end of the incubation time the reaction was stopped by the addition of 900 μ l of 0.1% trifluoroacetic acid and loaded on C18 column. HPLC analysis revealed that the peptide was completely dephosphorylated since under the experimental conditions used in this analysis, any remaining phosphorylated peptide would elute in fractions 46-48. No radioactivity was detected in fractions 40-55. However, total recovery of cpm injected to the column was obtained in fractions 7-9 (Fig. 11), indicating that PP1c dephosphorylation of the peptide was essentially complete.

HPLC analysis of peptide dephosphorylation was extended to include the dephosphorylation of phosphoS6-21 peptide by PP2Ac. S6-21 peptide (3.5 nmoles) was multisite phosphorylated by an S6/H4 kinase as described in Methods. The product was loaded on an HPLC reverse phase C18 column and

was eluted with a linear gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid. The phosphopeptide was eluted in fractions 43-60. Radioactivity detected consisted of one major peak in addition to approximately 3 minor peaks, confirming that the peptide was phosphorylated at more than one site (Fig. 12). To examine if PP2Ac dephosphorylates phosphoS6-21 at all sites, the peptide (3.5 nmoles) was incubated with PP2Ac (150 ng) and the dephosphorylation reaction was allowed to proceed for 60 min. Inorganic phosphate liberated was removed by ion exchange column as discussed in Methods. Radioactivity detected from an HPLC elution profile was less than the radioactivity observed with phosphoS6-21, indicating that the peptide was only partially dephosphorylated (Fig. 13).

To investigate the selectivity of PP2Ac, phosphoS6-21 (3.5 nmoles) was digested with 1.5% (w/w) thermolysin (180) and the products were analyzed on HPLC. Five major phosphorylated products designated A, B, C, D, E in addition to an undigested phospho S6-21, were obtained from the thermolysin digest of S6-21 phosphorylated by S6/H4 kinase (Fig. 14A). These five phosphorylated products eluted at acetonitrile concentration of 1, 8, 20, 24, and 30% respectively. Phosphopeptides A and C were dephosphorylated by PP2Ac by approximately 90% and peak B was dephosphorylated by more than 25% (Fig. 14B). Amino acid analysis and peptide sequencing of peak C predicts that peak C is composed of L-R-A-S-T-S-K-S-E-S-S-Q-K. The amino acid composition analysis of peak C is shown in Table VI. Under the conditions used in these experiments, the sequence of peak A could not be determined. However, other studies in this laboratory have shown that the sequence of phosphopeptide A is L-S-S (173,174). The overall dephosphorylation of

phospho S6-21 thermolysin digests by PP2Ac was about 50%. Similarly, phospho S6-21 was partially dephosphorylated by PP1c (~80 ng) which did not show any selectivity with respect to a specific thermolysin product (Fig. 15A, B).

Purification of PP1c and PP2Ac: Phosphoprotein phosphatases PP1c and PP2Ac were purified to apparent homogeneity as described by DeGuzman *et al.* (175). The purification procedure was conducted in the presence of protease inhibitors as discussed in Materials and Methods. The molecular weights of PP1 and PP2A catalytic subunits were 38 Kd, compared to 32 Kd for PP1c and PP2Ac purified in the absence of protease inhibitors.

During the purification process, phosphatase activity was assayed for using synthetic S6-21 peptide phosphorylated by a partially purified S6/H4 kinase. Two peaks of phosphatase activity were obtained at mean conductivities of 6 and 15 mmho when a DEAE-52 column was eluted with a linear salt gradient. The first peak contained PP1c activity and the second peak contained PP2Ac activity according to DeGuzman and Lee (175). The two phosphatase peaks were separately pooled and further purification proceeded for each peak separately. Both enzymes eluted off an S-200 column (100x2.5 cm) at a V_e/V_o of 1.5.

In order to confirm that the purified phosphatases from lymphosarcoma tumors correspond to PP1c and PP2Ac, both enzymes were examined for their phosphorylase phosphatase activities (Fig. 16). Both enzymes dephosphorylated phosphorylase α . PP1c activity was approximately 4-fold

higher than PP2Ac. In the presence of 2 µg/ml inhibitor I-2, PP1c activity was inhibited by approximately 90% whereas PP2Ac retained its full activity.

Rates of dephosphorylation of phosphoMLC(3-13) and phosphoS6-21 by PP1c and PP2Ac: To investigate the rates of dephosphorylation of the PKC-phosphorylated MLC(3-13) synthetic peptide with both PP1c and PP2Ac, and to determine whether the peptides were completely dephosphorylated by either enzyme, the phosphopeptide was incubated with either PP1c or PP2Ac as described in Methods. Excess amounts of PP1c (84 ng) or PP2Ac (150 µg) catalyzed complete dephosphorylation of the peptide. However, the rate of dephosphorylation by PP1c was much greater than that by PP2Ac (Fig. 17). In order not to underestimate the activities of both enzymes and to determine the initial rates of dephosphorylation, PP1c and PP2Ac were diluted 450- and 45-fold, respectively. Figure 18 illustrates the rate of dephosphorylation of phosphoMLC(3-13) by PP1c and PP2Ac. When the diluted enzymes were incubated with the phosphoMLC(3-13), it took 6 min for PP1c to liberate 50% of the phosphate content of the peptide, whereas 20 min was needed for PP2Ac to dephosphorylate the same peptide by 50% (Fig. 18). In addition, the dephosphorylation curve by the 450-fold-diluted PP1c was curvilinear compared to the linear dephosphorylation curve of the 45-fold-diluted PP1c. This demonstrates that although both enzymes completely dephosphorylate the peptide, the phosphoMLC(3-13) phosphatase activity of PP1c is more efficient than that of PP2Ac. When 50-fold-diluted PP1c or PP2Ac were incubated with gizzard phospholight chain (1.5 µmoles), neither enzyme was capable of

completely dephosphorylating the phosphoprotein. After 40 min of incubation with either enzyme, gizzard phospholight chains was dephosphorylated by 50% (Fig. 19). On the other hand, excess amounts of PP1c and PP2Ac (84 and 150 ng respectively) were incubated with the S6/H4 kinase-phosphorylated S6-21, neither enzyme was capable of completely dephosphorylating the peptide (Fig. 20). The dephosphorylation rate of PP2Ac during the first 3 min of incubation was 4-fold higher than than of PP1c (22 and 5.5% respectively). When either enzyme was incubated with the phosphopeptide for 60 min, the phosphopeptide was dephosphorylated by 50% (Fig. 20). These data demonstrate that PP1c and PP2Ac are more effective in dephosphorylating a protein kinase C-modified peptide than an S6/H4 kinase-modified peptide. This correlates precisely with what was observed with HPLC analyses of peptide dephosphorylation as described earlier.

Kinetic analysis of PP1c- and PP2Ac-catalyzed dephosphorylation of phosphopeptides: To better understand the rates of phosphopeptide dephosphorylation and to get an insight into the specific activities of PP1c and PP2Ac with the phosphopeptides, kinetic analyses were carried out at constant concentrations of PP1c and PP2Ac using a variety of phosphopeptide concentrations. With phosphoMLC(3-13), PP1c and PP2Ac concentrations (1.0 and 17 ng respectively) were selected so that during the incubation time peptide dephosphorylation does not exceed 5.4 and 1.4 pmol/min respectively. Different concentrations of phosphoMLC(3-13) ranging from 2.5 to 20 μ M were incubated with either PP1c or PP2Ac for 6 min as described in Methods. Figures 21 and 22 illustrate-double reciprocal plots of

PP1c- and PP2Ac-catalyzed dephosphorylation reactions as a function of phosphoMLC(3-13) concentrations. PP1c-catalyzed dephosphorylation of phosphoMLC(3-13) exhibited much higher reactivity than PP2Ac.

PhosphoMLC(3-13) had an apparent K_m with PP1c of 7.1 μM and a V_{max} of 24 $\mu\text{mol}/\text{min}\cdot\text{mg}$ (Table VII). With PP2Ac, the observed K_m and V_{max} were 18 μM and 0.77 $\mu\text{mol}/\text{min}\cdot\text{mg}$ respectively. The pseudo-first-order rate constant, V/K , for the PP1c-dephosphorylated peptide was 80-fold higher than that for PP2Ac-dephosphorylated peptide (Table VII).

In order to get an insight of the specificity of PP1c and PP2Ac, it was necessary to compare their specific activities toward the PKC-modified peptide (phosphoMLC(3-13)) with another peptide modified by a different kinase such as the S6/H4 kinase-modified S6-21. The amounts of PP1c and PP2Ac (84 and 150 ng respectively) used for this experiment were adjusted so that each enzyme-catalyzed phosphoS6-21 dephosphorylation does not exceed 20%. Different concentrations of phosphoS6-21 ranging from 50-250 μM were incubated with either PP1c or PP2Ac for 10 min as described in Methods. The PP1c- and PP2Ac- catalyzed dephosphorylation reactions as a function of phosphoS6-21 are illustrated in Figures 23 and 24. PhosphoS6-21 peptide had an apparent K_m with PP1c of 40 μM and a V_{max} of 0.26 $\mu\text{mol}/\text{min}\cdot\text{mg}$. With PP2A, the observed K_m and V_{max} were 127 μM and 0.98 $\mu\text{mol}/\text{min}\cdot\text{mg}$ respectively. The V/K for PP1c-dephosphorylated peptide was 0.13 $\text{min}^{-1}\cdot\text{mg}^{-1}$ whereas that for PP2A-dephosphorylated peptide was 0.15 $\text{min}^{-1}\cdot\text{mg}^{-1}$ (Table VII).

These data suggest that the PKC-modified phosphopeptide was a more favorable substrate for PP1c and PP2Ac than the S6/H4-modified peptide.

Characterization of PP1c and PP2Ac using phosphoMLC(3-13) and phosphoS6-21 as substrates: The effect of various inhibitors and activators on phosphoprotein phosphatases have been demonstrated (192-195). A variety of cations, such as Mn^{+2} , histone H1, protamine, and poly L-lysine have been shown to stimulate dephosphorylation of glycogen synthase by phosphoprotein phosphatases 1 and 2A (78, 104). On the other hand, various inhibitors such as okadaic acid and microcystin-LR have been shown to inhibit phosphorylase *a* dephosphorylation by PP1 and PP2A (58). However, the influence of a large number of these phosphoprotein phosphatase effectors is dependent on the enzyme form and the phosphosubstrate (104). In addition, some effectors have been known to be enzyme specific. For example, heparin has been shown to inhibit the dephosphorylation of phosphorylase *a* when dephosphorylated by PP1, but not PP2A (193).

In an attempt to differentiate between PP1c and PP2Ac, the effects of various reagents on PP1c and PP2Ac phosphopeptide phosphatase activities were investigated.

With phosphoMLC(3-13), the presence of 5 mM $MgCl_2$, an activator of MgATP-dependent PP1 with phosphorylase *a*, inhibited the phosphoMLC(3-13) phosphatase activity of PP1c by more than 40%, whereas it did not have any effect of PP2Ac activity (Fig. 25). The addition of 5 mM of $MnCl_2$, an *in vitro* activator of PP1, inhibited both PP1c and PP2Ac by 70 and 50% respectively. The non-specific inhibitor of phosphoprotein phosphatases, sodium fluoride (50 mM), inhibited both PP1c and PP2Ac by more than 90 and 80% respectively. Sodium *orthovanadate* (50 mM), a known tyrosine phosphatase

inhibitor, was as potent an inhibitor as the non-specific phosphatase inhibitor sodium fluoride (50 mM). It almost completely inhibited phosphoMLC(3-13) activity of PP1c. However, PP2Ac was inhibited by approximately 85% (Fig. 25). The addition of 50 mM of β -glycerophosphate, a non-specific phosphatase inhibitor, to the phosphoMLC(3-13) dephosphorylation reactions inhibited PP1c and PP2Ac by approximately 80 and 90% respectively. The mucopolysaccharide heparin (50 μ g/ml), an inhibitor of PP1-G with phosphorylase *a*, partially inhibited PP1c and PP2Ac (45 and 25% respectively). Protamine (25 μ g/ml), a polycation polypeptide, inhibited PP1c by 80% and PP2Ac by 60%. Likewise, the presence of 15 μ g/ml of the polyamine polypeptide poly L-lysine, a PP2A activator, inhibited PP1c and PP2Ac by 90 and 70% respectively. Micromolar concentrations of the naturally occurring products, microcystin-LR (1 μ M) and okadaic acid (1 μ M), potent inhibitors of PP1, PP2A and their catalytic subunits with phosphorylase *a* as a substrate, failed to completely inhibit either PP1c or PP2Ac with phosphoMLC(3-13) as a substrate. The physiological inhibitor of PP1, I-2 (2 μ g/ml), partially inhibited PP1c and did not have any effect on PP2Ac when phosphoMLC(3-13) was used as a substrate (Fig. 25).

In order to determine whether phosphopeptide substrates play a role in the selectivity of phosphoprotein phosphatase effectors, the effects of the inhibitors and activators with the same concentrations described above for PP1c and PP2Ac were examined with phosphoS6-21 as a substrate. MgCl_2 (5 mM) partially inhibited PP1c and PP2Ac. The same concentration of MnCl_2 inhibited PP2Ac by 34% and activated PP1c by more than 60% (Fig. 27). Sodium fluoride inhibited PP1c and PP2Ac by 100 and 92% respectively.

Sodium *orthovanadate* and β -glycerophosphate inhibited phospho S6-21 activity of PP1c by about 70 and 80% respectively, and that of PP2Ac by about 80 and 90% respectively. The presence of heparin in the reaction mixture increased the phospho S6-21 activity of PP1c by more than 300% and that of PP2Ac by 13%. Both enzymes were partially inhibited by the peptide poly L-lysine. The natural products microcystin-LR and okadaic acid (1 μ M each) completely inhibited both phosphatases. The physiological inhibitor I-2 inhibited 90% of PP1c activity and slightly increased PP2Ac activity when phospho S6-21 was used as a substrate (Fig. 26).

Dose-dependent inhibition of PP1c and PP2Ac by microcystin-LR and okadaic acid: The naturally occurring cyclic heptapeptide toxin, microcystin-LR and the non-phorbol ester tumor promoter toxin, okadaic acid have been described to be very potent inhibitors of PP1 and PP2A. A number of reports have demonstrated that higher concentrations of microcystin-LR and okadaic acid are needed to inhibit PP1 than those required to inhibit PP2A (50-62).

High concentrations (1 μ M) of microcystin-LR and okadaic acid partially inhibited phosphoMLC(3-13) dephosphorylation by PP1c and PP2Ac. However, with phosphoS6-21, PP1c and PP2Ac were inhibited by 100% (as reported above). To examine the concentrations of microcystin-LR and okadaic acid required to inhibit PP1c and PP2Ac with phosphoS6-21, different inhibitor concentrations ranging from 1.0 pM to 10 μ M for microcystin-LR and 1.0 pM to 1.0 μ M for okadaic acid were separately incubated with either PP1c or PP2Ac. IC₅₀ (50% inhibition) of PP1c and PP2Ac by microcystin-LR was 30 and 1 nM

respectively (Fig. 27), whereas 50% inhibition of PP1c and PP2Ac was achieved by okadaic acid concentrations of 40 and 3.0 nM respectively (Fig. 28). In agreement with data obtained with protein substrates, these data demonstrate that PP2Ac is more sensitive to these toxins than PP1c.

Collectively, these data demonstrate that the utilization of synthetic peptides as substrates for PP1c and PP2Ac has proven useful in providing an insight into the specificity determinants of these enzymes. PP2Ac dephosphorylated two sites out of several sites phosphorylated by S6/H4 kinase. PP1c did not show any specificity in dephosphorylating the phosphopeptides. PP1c and PP2Ac are more efficient in dephosphorylating protein kinase C-modified substrates compared to those modified by S6/H4 kinase. While PP2Ac displays selectivity in dephosphorylating the S6/H4 kinase-modified substrate, PP1c did not show any selectivity in dephosphorylating the same substrate. Furthermore, the effects of different inhibitors and activators on PP1c and PP2Ac were substrate-dependent. With phosphoMLC(3-13), MgCl_2 (5 mM) inhibited PP1c and PP2Ac. Under the same experimental conditions, MgCl_2 inhibited PP1c and did not have any effect on PP2Ac when phosphoS6-21 peptide was used as a substrate. Heparin inhibited PP2Ac with both substrates. On the other hand, heparin activated PP1c with phosphoS6-21 and inhibited it with phosphoMLC(3-13) as a substrate. With either substrate, the physiological inhibitor I-2 inhibited PP1c and slightly stimulated PP2Ac when phosphoS6-21 was the substrate. PP2Ac appeared to be more sensitive to microcystin-LR and okadaic acid. Finally, the P81 paper assay for peptide dephosphorylation has proven accurate and reliable in determining peptide dephosphorylation by PP1c and PP2Ac.

TABLE I

Effect of Protein Kinase C Activators on the Phosphorylation of MLC(3-13) and MLC(5-13) as Determined with Lipid Dispersions

Effector	Protein kinase C activity (<u>pmol phosphate incorporated/min</u>)	
	MLC(3-13)	MLC(5-13)
None	0.9	0.7
CaCl ₂ (50 μ M)	1.3	3.1
OAG	4.8	3.4
PS	3.0	3.9
CaCl ₂ + OAG	7.4	4.2
CaCl ₂ + PS	12.2	9.4
OAG + PS	15.7	11.1
CaCl ₂ + OAG + PS	31.1	22.1

Protein kinase C (60 ng) was incubated with 0.1 M MgCl₂, [γ -³²P]ATP (296 dpm/pmol) and 200 μ M MLC(3-13) or MLC(5-13) in the presence or absence of effectors. Phosphopeptide was quantitated as described in Methods. When added, the amounts of OAG and PS present in the assay mixture were 0.175 and 5 μ g respectively.

TABLE II

Effect of Protein Kinase C Activators on the Phosphorylation of MLC(3-13) and MLC(5-13) as Determined by the Micellar Assay Procedure

Effector	Protein kinase C activity (pmol phosphate incorporated/min)	
	MLC(3-13)	MLC(5-13)
None	1.6	0.9
CaCl ₂	2.0	1.9
OAG	1.8	2.1
PS	2.1	1.9
CaCl ₂ + OAG	4.5	3.5
CaCl ₂ + PS	4.6	4.5
OAG + PS	2.9	2.2
CaCl ₂ + OAG + PS	43	27

Protein kinase C (60 ng) was incubated with 0.1 M MgCl₂, [γ -³²P]ATP (108 cpm/pmol) and 200 μ M MLC(3-13) or MLC(5-13) in the presence or absence of effectors. Phosphopeptide was quantitated as described in Methods. When added, the amounts of OAG and PS present in the assay mixture were 2 mol% and 10 mol% respectively.

TABLE III
Effect of the Order of Binding of Protein Kinase C
to Phosphatidyl Serine and OAG

<u>Step 1</u>		<u>Step 2</u>		<u>Protein kinase C activity</u>	
<u>Effector</u>	<u>Incubation^a</u> (5 min)	<u>Effector</u>	<u>Incubation</u> (5 min)	<u>pmol/min</u>	
				<u>MLC(3-13)</u>	<u>MLC(5-13)</u>
OAG	+	PS	-	40.0	24.2
PS	+	OAG	-	44.5	28.5
OAG	-	PS	+	43.7	27.9

Protein kinase C (60 ng) was incubated with 0.1 M MgCl₂, [γ -³²P]ATP and 200 μ M MLC(3-13) or MLC(5-13) in the presence of 10 mol% PS and 2 mol% OAG. Phosphopeptide was quantitated as described in Methods.

^a +, The effector was incubated for 5 min in the reaction mixture before starting the reaction with Mg[γ -³²P]ATP. -, No incubation was involved.

TABLE IV
Effect of Buffer on the Phosphatase Assay Background

Buffer ^a	Background					
	dpm	pmol	% total ^b	dpm	pmol	% total
	[S6-21]					
	140 pmol/paper			350 pmol/paper		
None	290	0.18	3.8	415	0.25	3.1
Tris (pH 7.5)	220	0.13	2.9	580	0.35	3.9
MES (pH 6.8)	370	0.22	4.8	470	0.29	3.1
NaH ₂ PO ₄ (pH 7.0)	275	0.17	3.6	525	0.32	3.4
HEPES (pH 7.0)	320	0.20	4.1	380	0.23	2.6
Imidazole (pH 6.5)	340	0.21	4.4	390	0.24	2.6
β-glycerophosphate (pH 6.8)	325	0.51	4.1	550	0.34	3.7

^a All buffers were 20 mM.

^b Phosphopeptide was quantitated by counting the P81 papers and total recovered radioactivity was determined by adding the background radioactivity and the phosphopeptide radioactivity.

TABLE V

Effect of Inhibitors and Activators on the Phosphatase Assay Background

Effector ^a	Background		
	dpm	pmol	% total ^b
None	343	0.21	2.9
MgCl ₂ (10 mM)	364	0.22	2.9
MgCl ₂ (50 mM)	362	0.22	2.9
MnCl ₂ (50 mM)	463	0.28	3.7
NaF (50 mM)	388	0.23	3.1
Na ₃ VO ₄ (50 mM)	409	0.25	3.2
Na ₂ HPO ₄ (100 mM)	349	0.21	2.9
Microcystin-LR (2 μ M)	348	0.21	2.8

^a All effectors were dissolved in 20 mM TrisCl, pH 7.5. The [S6-21] applied to each paper was 350 pmol.

^b Phosphopeptide was quantitated by counting the P81 papers and total recovered radioactivity was determined by adding the background radioactivity and the phosphopeptide radioactivity.

TABLE VI
Amino Acid Composition of Phosphopeptide C

Amino Acid Residue	Mole Ratio
Asx	N.D.
Thr	0.99
Ser	3.75
Glx	2.28
Pro	N.D.
Gly	N.D.
Ala	0.32
Cys	N.D.
Val	N.D.
Met	N.D.
Ile	N.D.
Leu	1.00
Tyr	N.D.
Phe	N.D.
Lys	3.44
His	N.D.
Trp	N.D.
Arg	2.36

N.D., not detected.

Phosphopeptide C (120 pmol) was hydrolyzed at 116°C for 15 min in 6 N HCl in vacuo as described by Westall and Hesser (181). Amino acid detection was by post column derivatization with orthophthaldehyde (182).

TABLE VII

Kinetic Constants for the Dephosphorylation of PhosphoMLC(3-13)
and PhosphoS6-21 Synthetic Peptides by PP1c and PP2Ac

	PP1c ^a		PP2Ac ^a	
	MLC(3-13) ^b	S6-21 ^b	MLC(3-13) ^b	S6-21 ^b
Km (μM)	7.1	40.0	18	127
Vmax ($\mu\text{mol}/\text{min}/\text{mg}$)	24	0.26	0.77	0.98
V/Km ($\text{min}^{-1}\cdot\text{mg}^{-1}$)	67.6	0.13	0.85	0.15

^a PP1c (~1.0 ng) and PP2Ac (~17 ng) were assayed with MLC(3-13) at concentrations ranging from 2.5 μM to 20 μM . PP1c (~80 ng) and PP2Ac (~150 ng) were assayed with S6-21 at concentrations ranging from 50 μM to 250 μM .

^b Sequences for both peptides are shown in Methods.

Fig. 1. Calcium Dependence of Protein Kinase C Activation. Protein kinase C (60 ng) was incubated for 10 min at 30°C with 0.1 M MgCl₂, and [α -³²P]ATP in the presence of 10 mol% PS and 2 mol% OAG with micellar assay or 5 μ g PS and 0.175 μ g OAG with the standard assay. CaCl₂ final concentrations ranged from 5 μ M to 100 μ M. Phosphopeptide was quantitated as described in Methods. MLC(3-13) concentration was 200 μ M. Assay was conducted by the standard and micellar procedures.

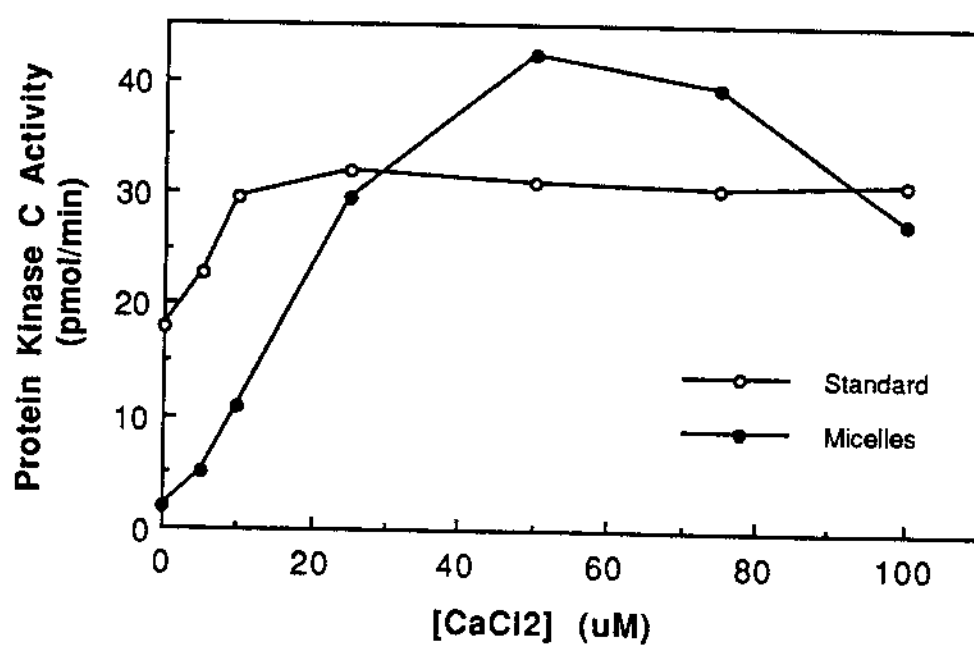


Fig. 2. Double Reciprocal Plot of OAG Dependence of Protein Kinase C Activation. Protein kinase C (60 ng) was incubated for 10 min at 30°C with 0.1 M MgCl₂, [γ -³²P]ATP and 200 μ M of **A**, MLC(3-13) and **B**, MLC(5-13) in the presence of 50 μ M CaCl₂. Micelles containing 10 mol% PS and OAG concentrations ranging from 0 to 4 mol% were used. Phosphopeptides were quantitated as described in Methods.

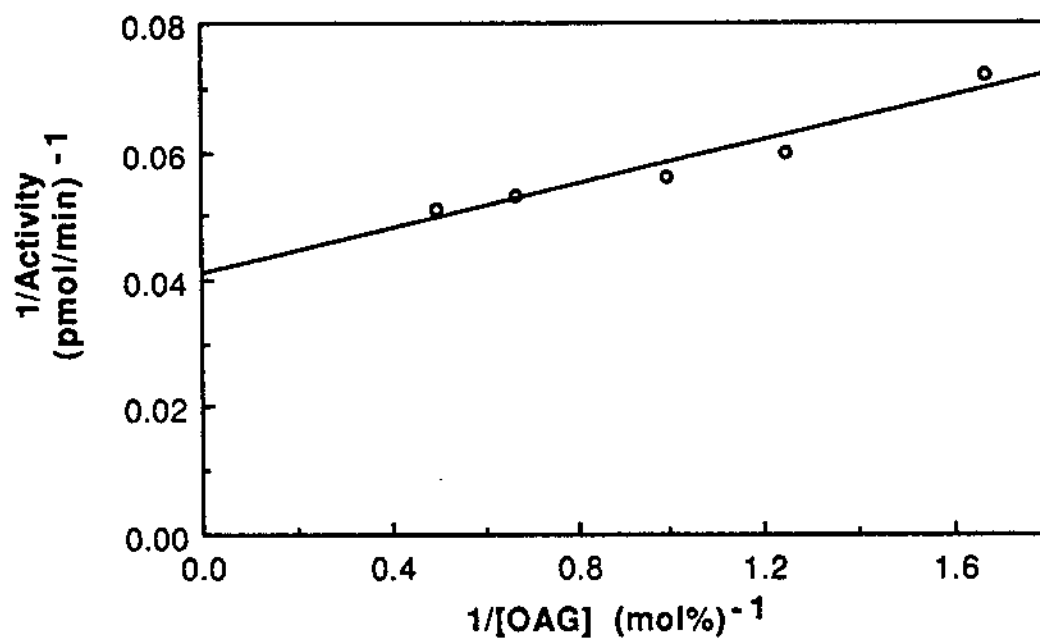
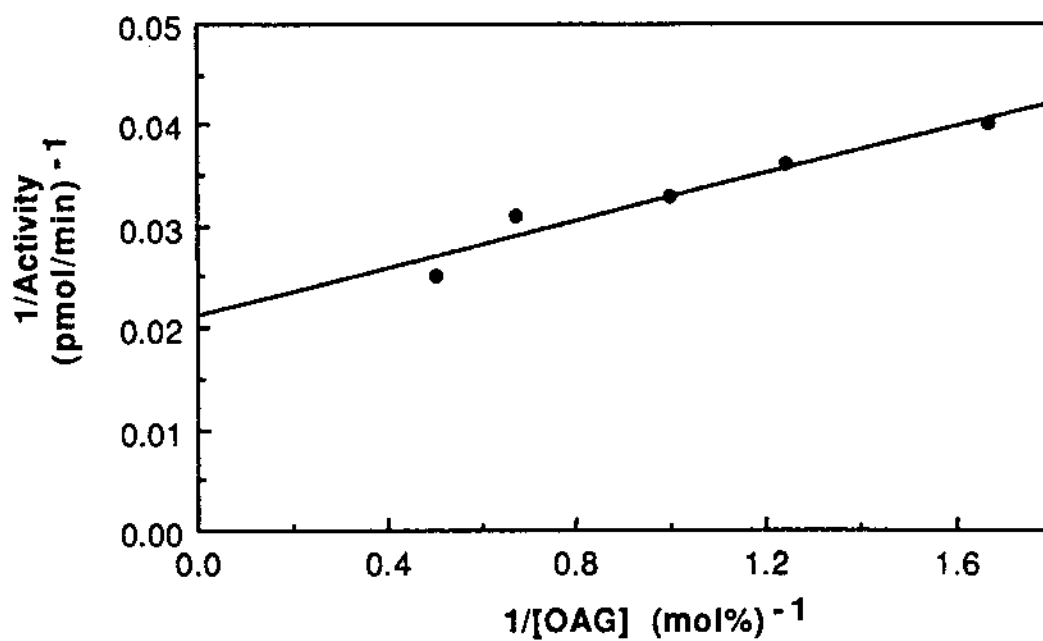
A. MLC(5-13)**B. MLC(3-13)**

Fig. 3. Phosphatidyl Serine Dependence of Protein Kinase C

Activation. Protein kinase C (60 ng) was incubated for 10 min at 30°C with 0.1 M MgCl₂, [γ -³²P]ATP and 200 μ M MLC(3-13) or MLC(5-13) in the presence of 50 μ M CaCl₂. Micelles containing 2 mol% OAG and PS concentrations ranging from 0 to 20 mol% were used. Phosphopeptide was quantitated as described in Methods.

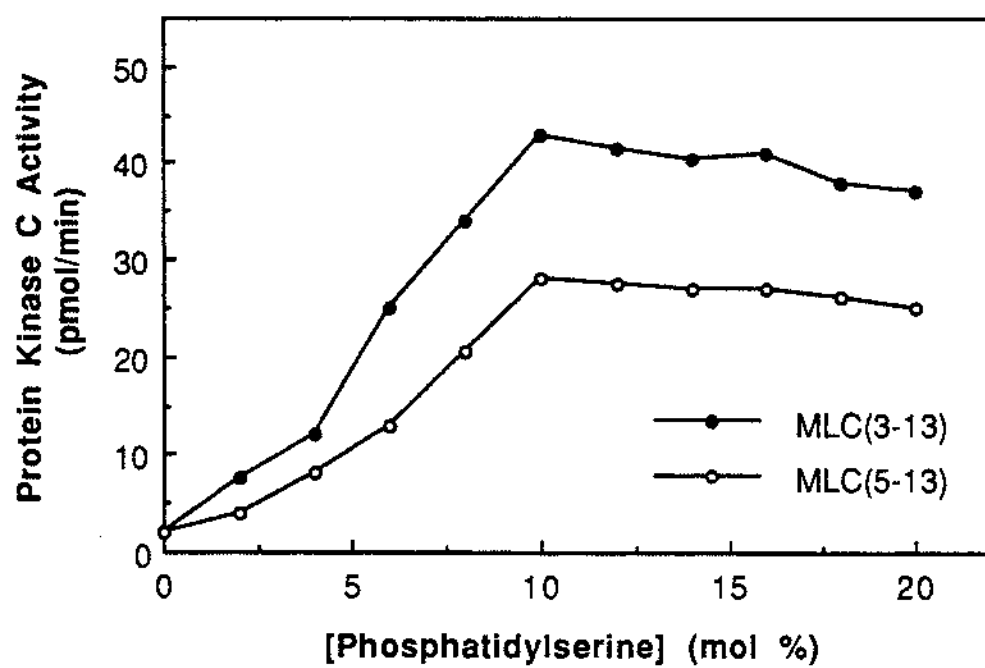
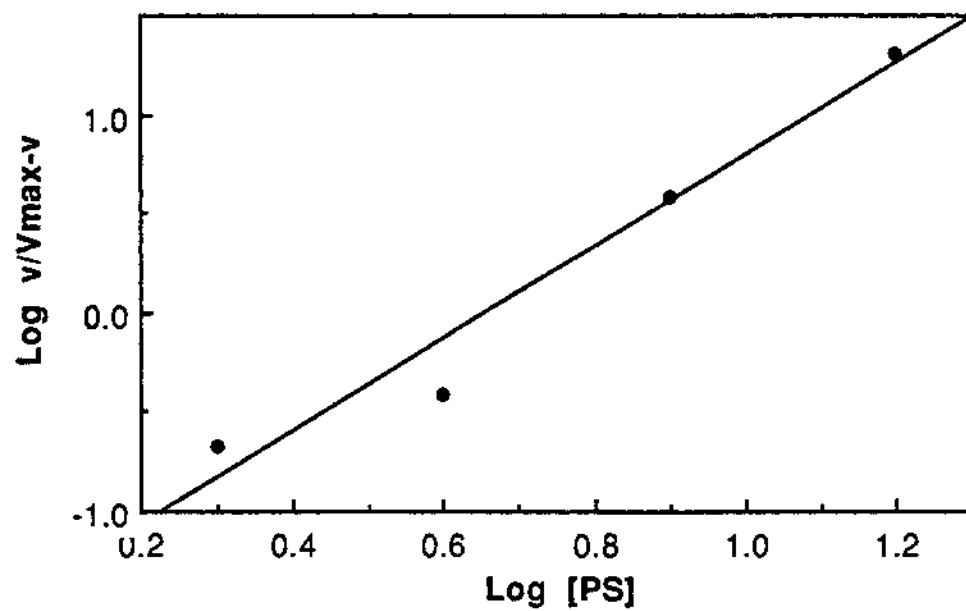


Fig. 4. Hill's Plot of the Number of Phosphatidyl Serine Molecules Bound to Protein Kinase C. Transformed data were obtained from Figure 3.

A. MLC(3-13)



B. MLC(5-13)

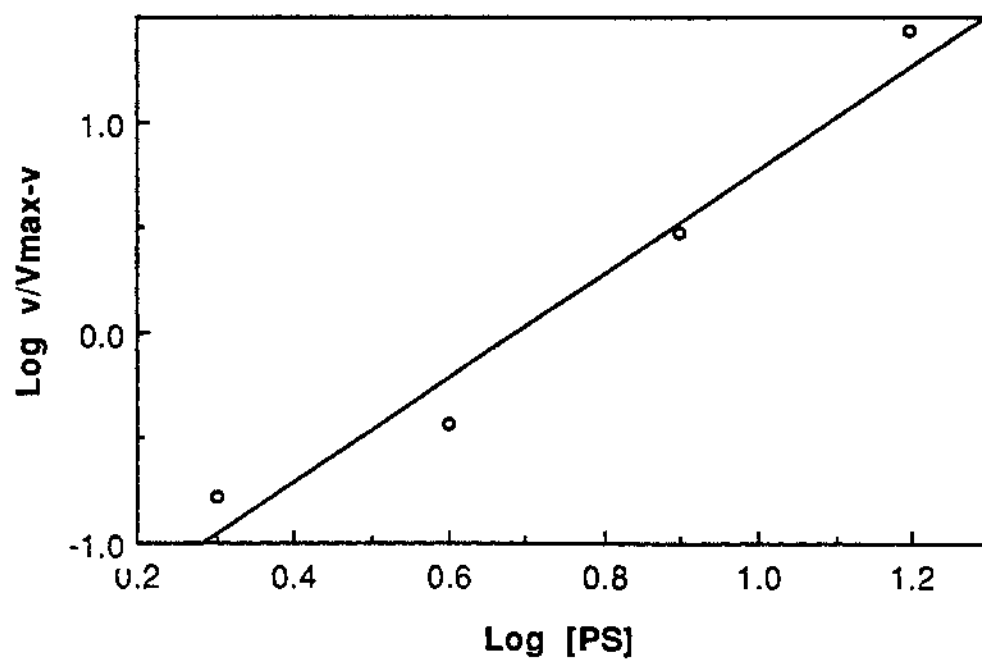


Fig. 5. PP1c Activity as Determined by the P81 Paper Assay and the Ion Exchange Assay. Rabbit muscle PP1c was incubated with 7 μ M (32 P)S6-21 (1630 dpm/pmol) for the designated time intervals as described in Methods. The (32 P)phosphate released from the peptide was quantitated by the P81 method (-*-) described in Methods or the AG 50W ion exchange method (-o-) described by McNall and Fischer (170).

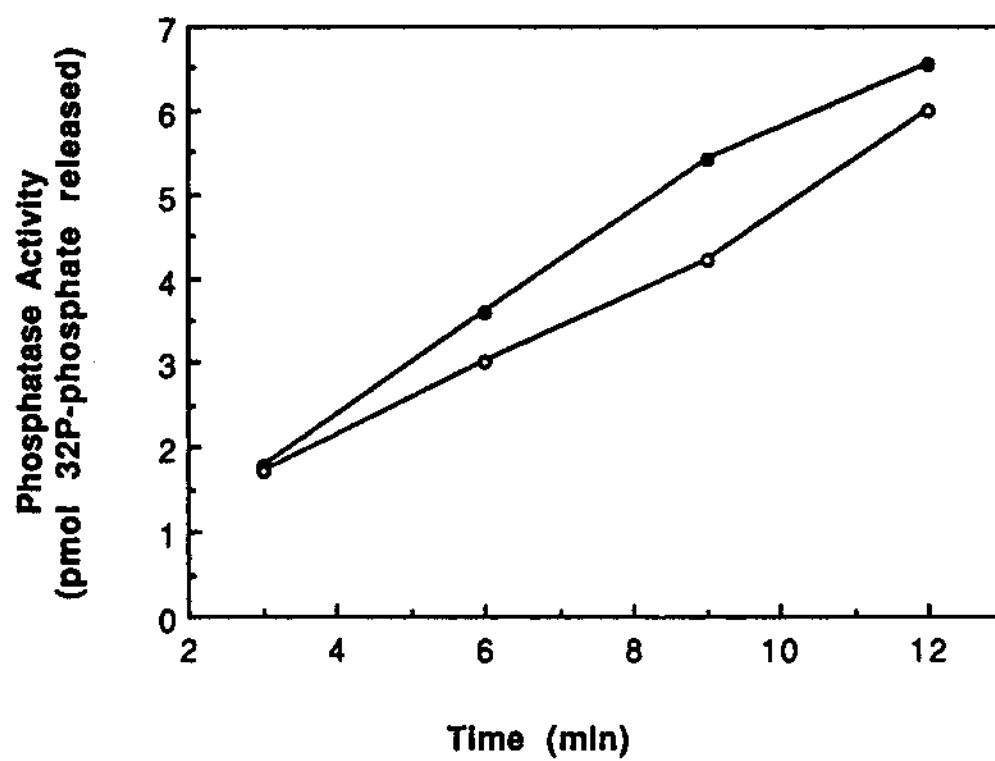


Fig. 6. Effect of Substrate Concentration on Assay Background. $(^{32}\text{P})\text{S6-21}$ (1630 dpm/pmol) corresponding to assay concentrations ranging from 0.7 μM to 7 μM was applied to P81 circles and washed with sodium phosphate as described in Methods. Radioactivity in the wash (background, -o-) and bound to the paper [$(^{32}\text{P})\text{S6-21}$, -•-] was quantitated by liquid scintillation counting. % total recovery is the amount of radioactivity recovered in the wash divided by the total radioactivity recovered in the wash and on the P81 paper.

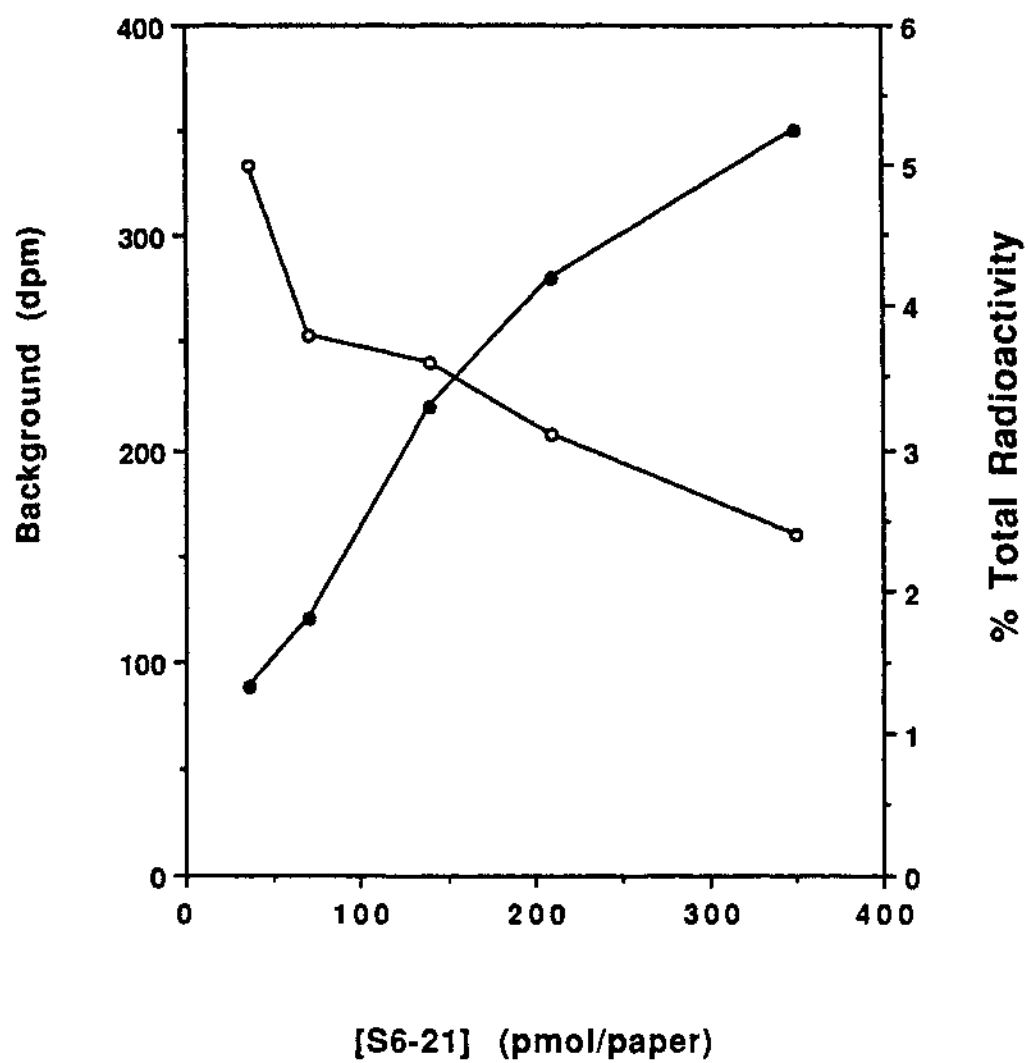


Fig. 7. The Effect of Wash Time on Phosphatase Assay

Background. (^{32}P)S6-21 (700 pmol, 2450 dpm/pmol) in 20 mM TrisCl, pH 7.5, was applied to P81 paper circles and washed in NaH_2PO_4 as described in Methods. Wash time varied from 1 min to 20 min.

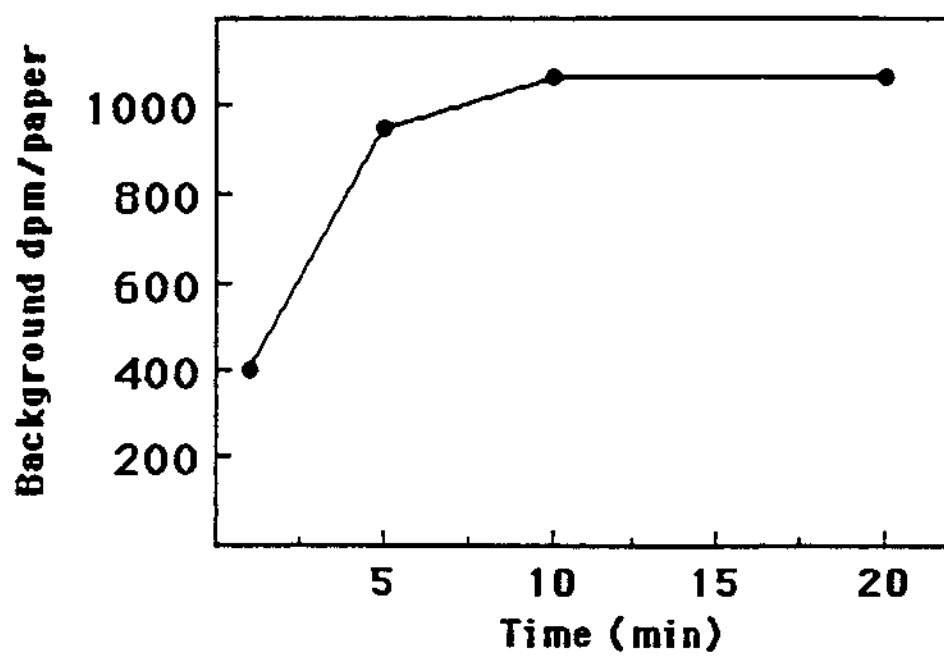


Fig. 8. HPLC Analysis of MLC(3-13) Phosphorylated by Protein Kinase C. MLC(3-13) (1.5 nmoles) was phosphorylated by protein kinase C (0.6 μ g) as described in Methods. Incubation was stopped by the addition of acetic acid to a final concentration of 30%, and [γ - 32 P]ATP was removed by AG 1x8 chromatography. The phosphopeptide was analyzed by reverse phase HPLC as described in Methods. Radioactivity in 20 μ l of the 0.5 ml fractions was determined.

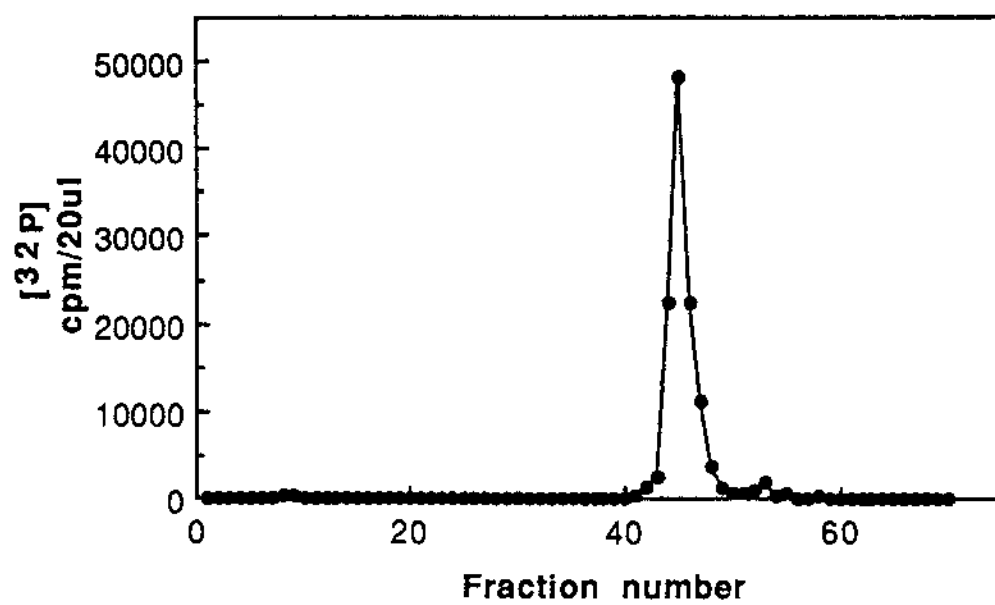


Fig. 9. HPLC Analysis of Partially Dephosphorylated MLC(3-13) by PP1c. PhosphoMLC(3-13) (0.3 nmoles) was dephosphorylated by PP1c (1 ng) for 6 min at 30°C as described in Methods. Dephosphorylation reaction was stopped by the addition of 900 µl 0.1% TFA. The dephosphorylation of the phosphopeptide was analyzed by reverse phase HPLC as described in Methods. Radioactivity in 20 µl aliquots of the 0.5 ml fractions was determined.

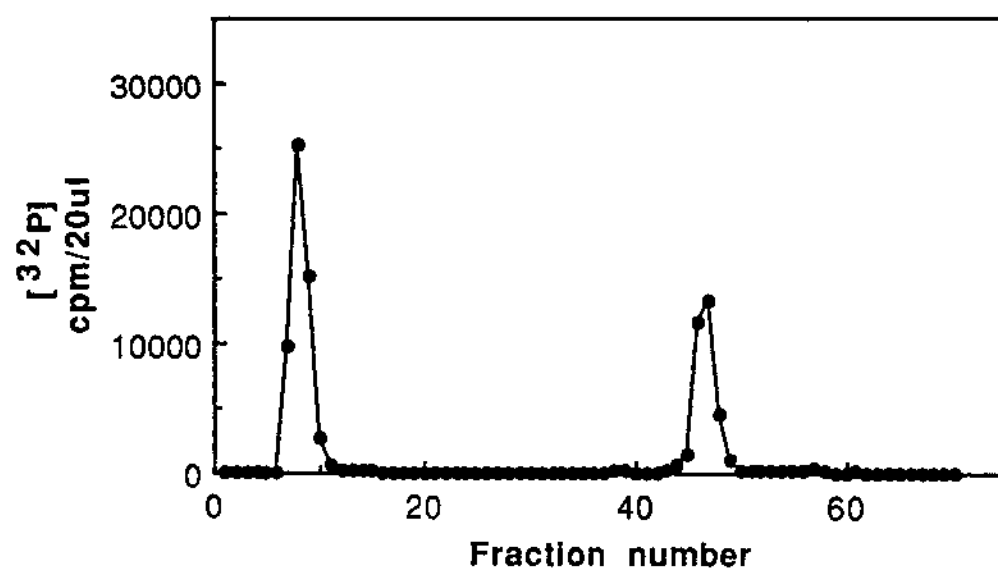


Fig. 10. HPLC Analysis of Partially Dephosphorylated MLC(3-13) by PP2Ac. PhosphoMLC(3-13) (0.3 nmoles) was dephosphorylated by PP2Ac (17 ng) for 20 min at 30°C as described in Methods. Dephosphorylation reaction was stopped by the addition of 900 μ l 0.1% TFA. The dephosphorylation of the phosphopeptide was analyzed by reverse phase HPLC as described in Methods. Radioactivity in 20 μ l aliquots of the 0.5 ml fractions was determined.

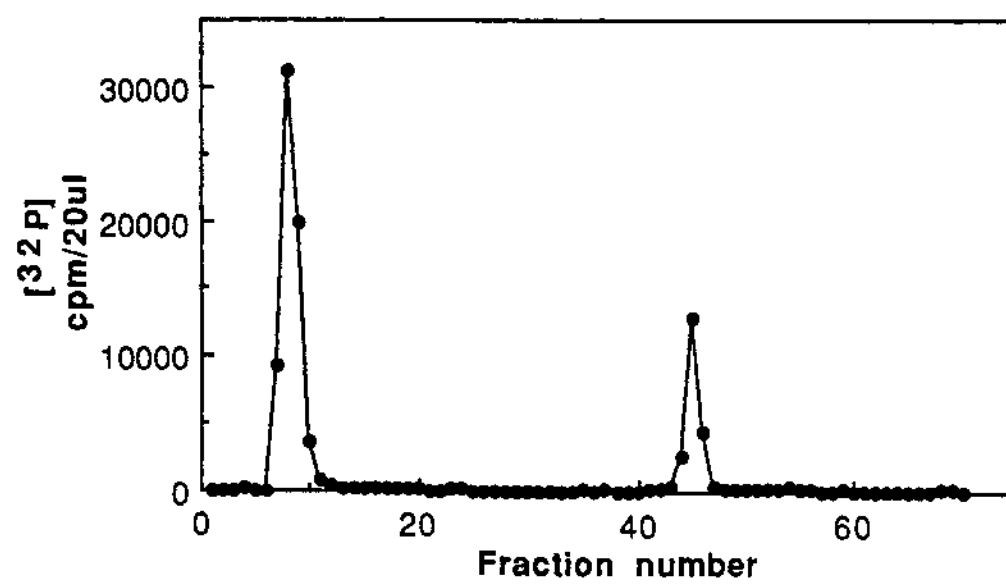


Fig. 11. HPLC Analysis of MLC(3-13) Completely Dephosphorylated by PP1C. PhosphoMLC(3-13) (0.3 nmoles) was completely dephosphorylated by PP1c (1 ng) as described in Methods. Incubation was stopped by the addition of 900 μ l 0.1% TFA. The phosphate liberated was analyzed by reverse phase HPLC as described in Methods. Radioactivity was determined in 20 μ l aliquots of the 0.5 ml fractions.

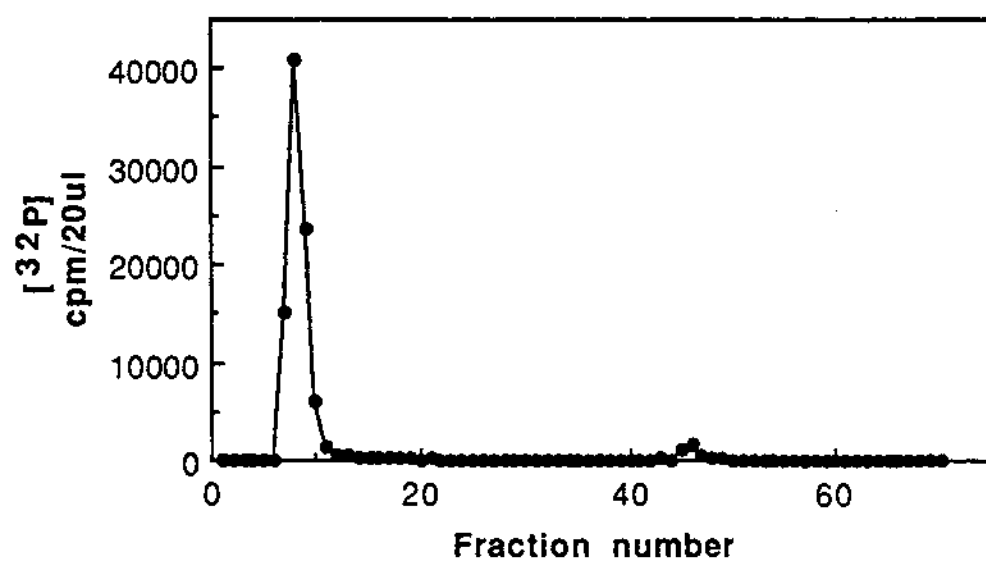


Fig. 12. HPLC Analysis of S6-21 Phosphorylated by S6/H4 Kinase.

S6-21 (3.5 nmoles) was phosphorylated by S6/H4 kinase (~100 µg) as described in Methods. Incubation was stopped by the addition of 900 µl 0.1% TFA. The phosphate liberated was analyzed by reverse phase HPLC as described in Methods. Radioactivity was determined in 20 µl aliquots of the 0.5 ml fractions.

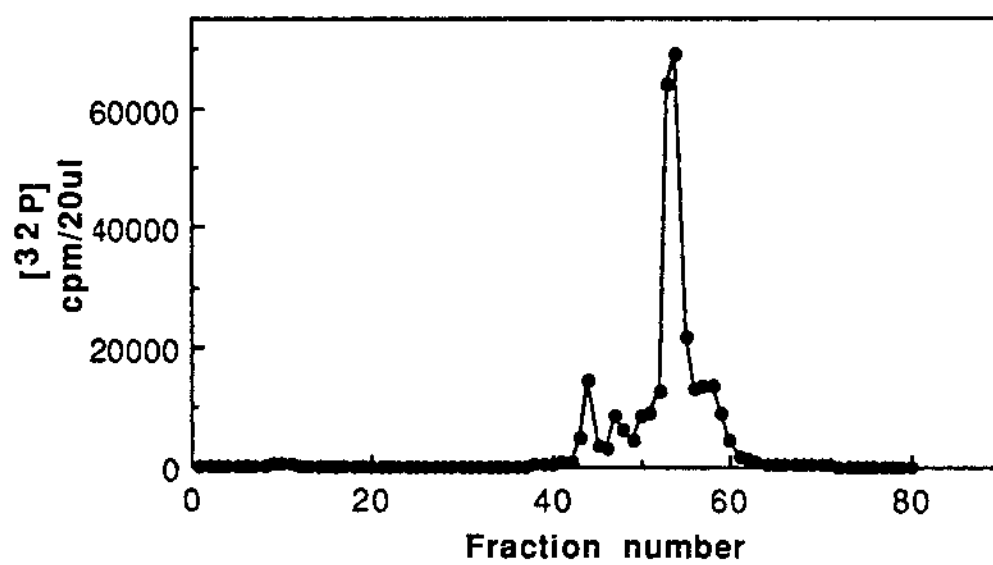


Fig. 13. HPLC Analysis of Partially Dephosphorylated S6-21 by PP2Ac. PhosphoS6-21 (3.5 nmoles) was dephosphorylated by PP2Ac (150 ng) for 60 min at 30°C as described in Methods. The phosphopeptide was analyzed by reverse phase HPLC as described in Methods. Radioactivity in 20 μ l aliquots of the 0.5 ml fractions was determined.

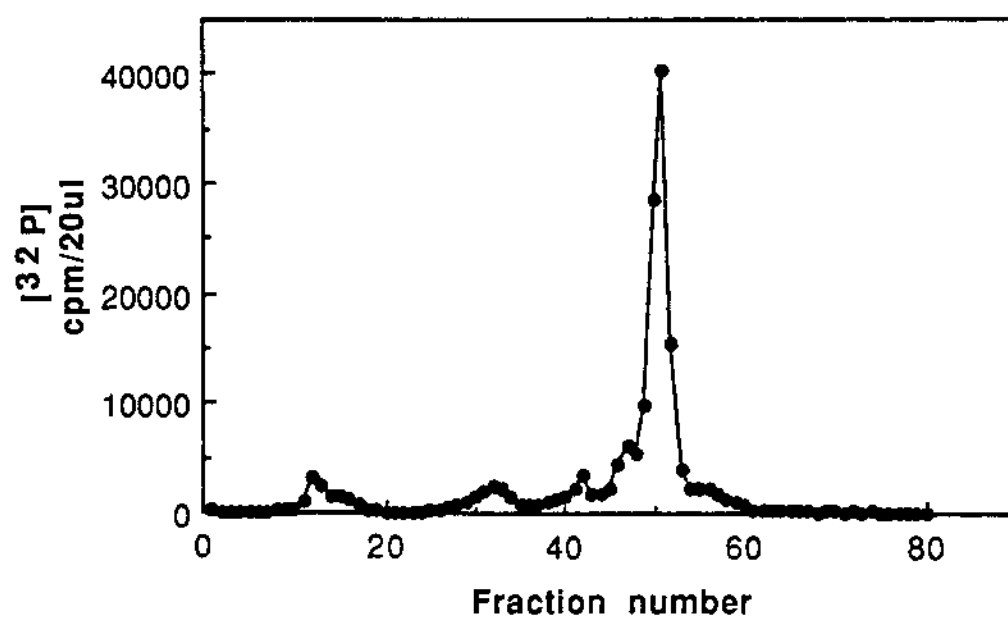


Fig. 14. HPLC Analysis of Phosphopeptides Obtained by Digestion of PhosphoS6-21 with Thermolysin. A, Thermolysin digest. S6-21 (3.5 nmoles) was phosphorylated by S6/H4 kinase and the product was digested by 1.5% (w/w) thermolysin as described in Methods. The phosphopeptides were analyzed by reverse phase HPLC eluted with 0-60% acetonitrile containing 0.1% TFA. **B, Site analysis of PP2Ac-catalyzed dephosphorylation of phosphoS6-21.** Phosphopeptides of the thermolysin digest were incubated with PP2A (150 ng) for 60 min at 30°C. Incubation was stopped by the addition of acetic acid to a final concentration of 30% and the liberated phosphate was removed by AG 1x8 chromatography. The product was lyophilized and analyzed by reverse phase HPLC as described in Methods.

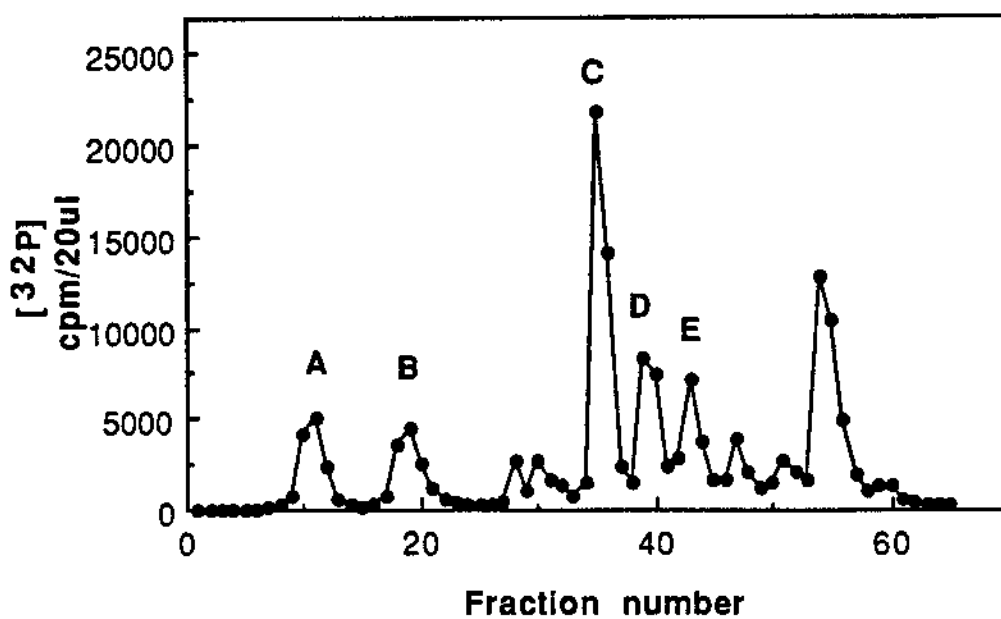
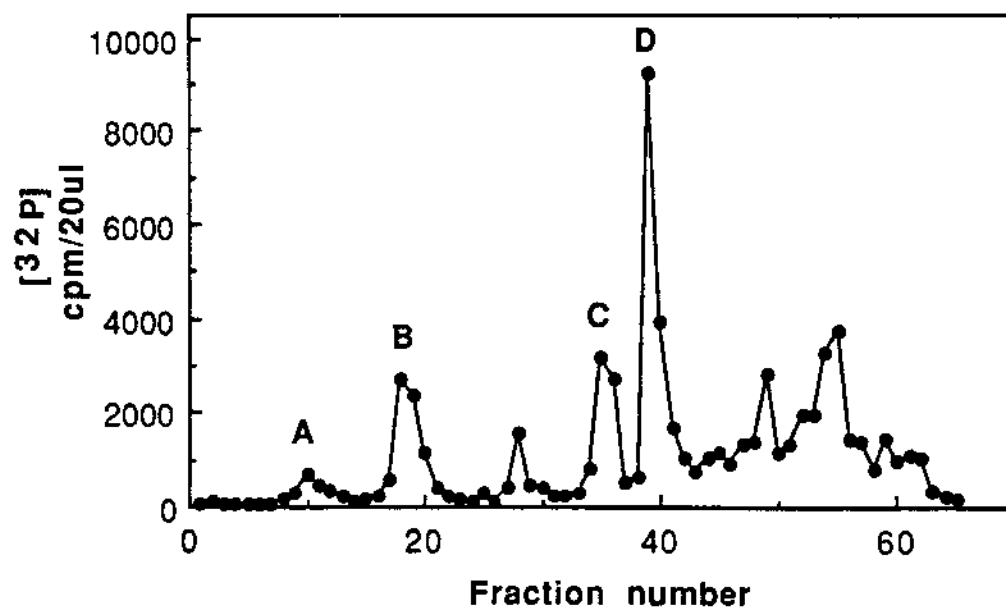
A. PhosphoS6-21**B. PP2Ac-dephosphorylated S6-21**

Fig. 15. HPLC Analysis of Phosphopeptides Obtained by Digestion of PhosphoS6-21 with Thermolysin. A, thermolysin digest. S6-21 (3.5 nmoles) was phosphorylated by S6/H4 kinase and the product was digested by 1.5% (w/w) thermolysin as described in Methods. The phosphopeptides were analyzed by reverse phase HPLC eluted with 0-60% acetonitrile containing 0.1% TFA. **B, site analysis of PP1c-catalyzed dephosphorylation of phosphoS6-21.** Phosphopeptides of the thermolysin digest were incubated with PP1c (80 ng) for 60 min at 30°C. Incubation was stopped by the addition of acetic acid to a final concentration of 30% and the liberated phosphate was removed by AG 1x8 chromatography. The product was lyophilized and analyzed by reverse phase HPLC as described in Methods.

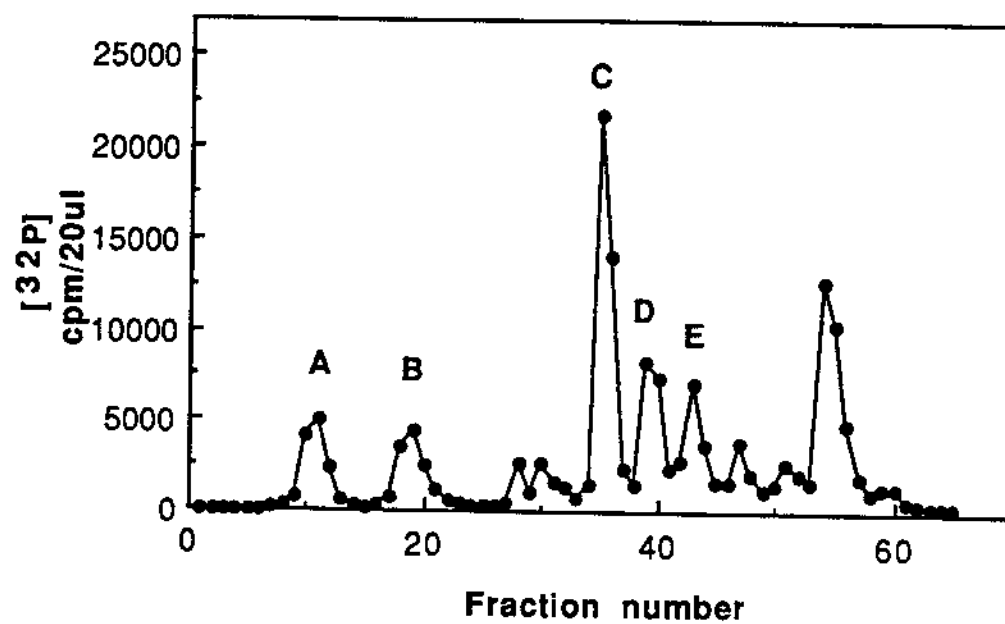
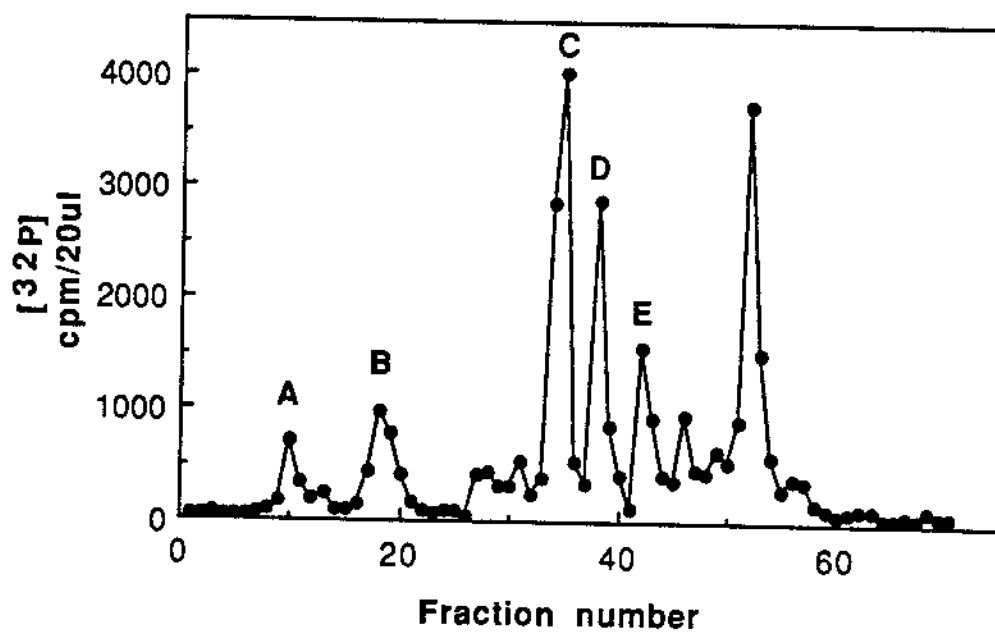
A. PhosphoS6-21**B. PP1c-dephosphorylated S6-21**

Fig. 16. Effect of Inhibitor-2 on the Phosphorylase Phosphatase Activity of PP1c and PP2Ac. Inhibitor-2 (2 $\mu\text{g/ml}$) was incubated with either PP1c and PP2Ac prior to the addition of phosphorylase *a*. PP1c and PP2Ac activities were quantitated by spotting 42ul of the reaction mixture onto P81 papers. The papers were washed several times with 66% ethanol and once with acetone. Radioactive glycogen on the dried paper was quantitated by liquid scintillation counting.

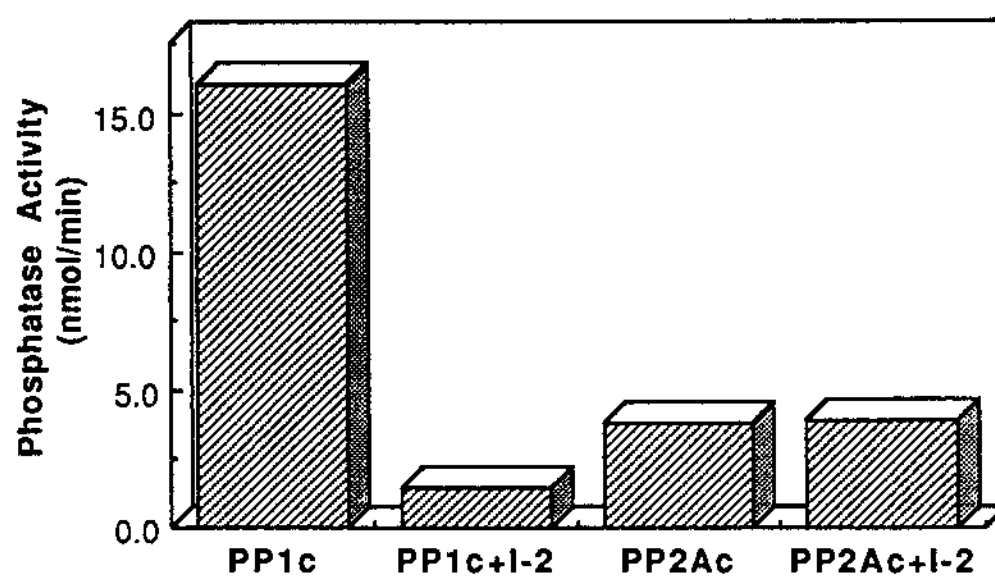


Fig. 17. Rate of Dephosphorylation of PhosphoMLC(3-13) by PP1c and PP2Ac. PhosphoMLC(3-13) (0.3 nmoles) was dephosphorylated by either PP1c (80 ng) or PP2Ac (150 ng) as described in Methods. Inorganic phosphate liberated was quantitated by the P81 paper assay procedure as described in Methods.

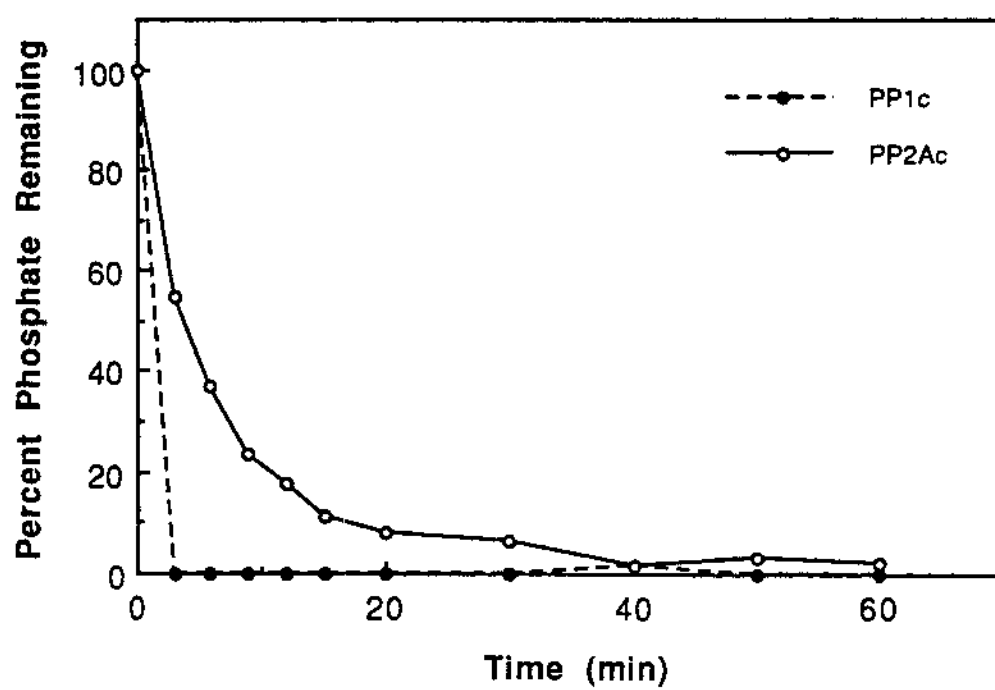


Fig. 18. Rate of Dephosphorylation of PhosphoMLC(3-13) by Diluted PP1c and PP2Ac. PhosphoMLC(3-13) (0.3 nmoles) was dephosphorylated by either PP1c (1 ng) or PP2Ac (17 ng) as described in Methods. Inorganic phosphate liberated was quantitated by the P81 paper assay procedure as described in Methods.

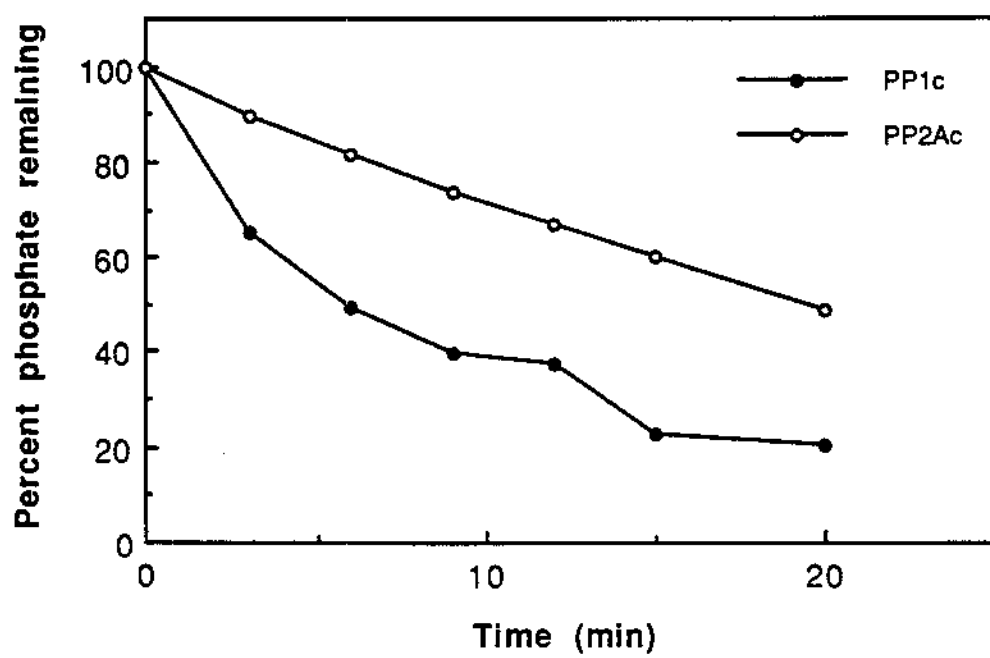


Fig. 19. Rate of Dephosphorylation of Gizzard Phospholight Chains by PP1c and PP2Ac. Gizzard phospholight chains (1.5 μ moles) were dephosphorylated by either PP1c (8 ng) or PP2Ac (15 ng) as described in Methods. Inorganic phosphate liberated was quantitated as described in Methods.

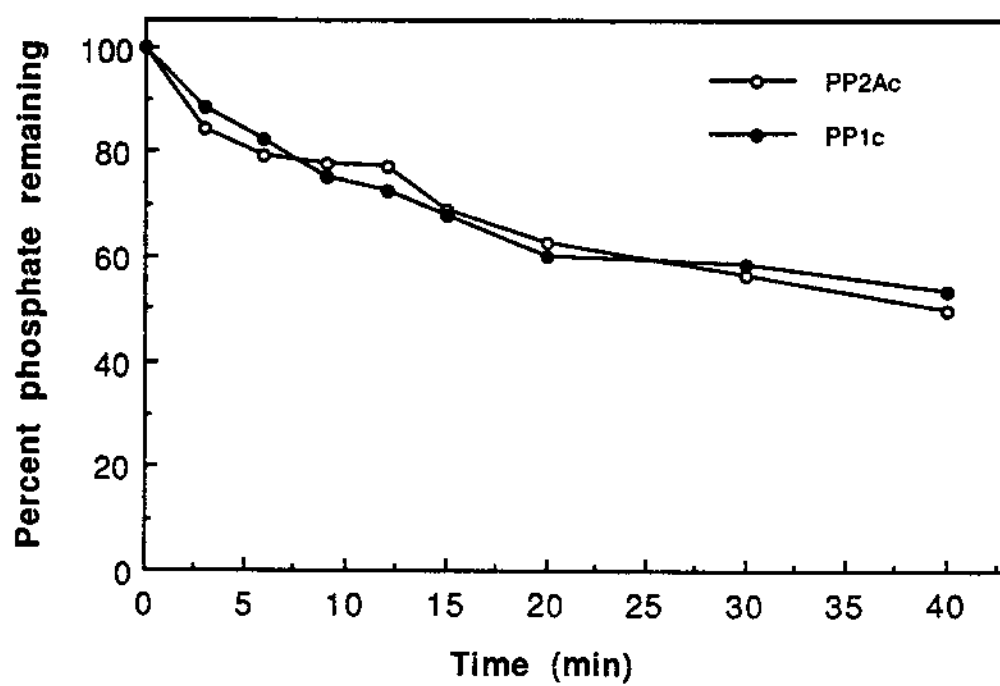


Fig. 20. Rate of Dephosphorylation of PhosphoS6-21 by PP1c and PP2Ac. PhosphoS6-21 (3.5 nmoles) was dephosphorylated by either PP1c and PP2Ac (0.2 and 0.375 μ g respectively) as described in Methods. Inorganic phosphate liberated was quantitated by the P81 paper assay procedure as described in Methods.

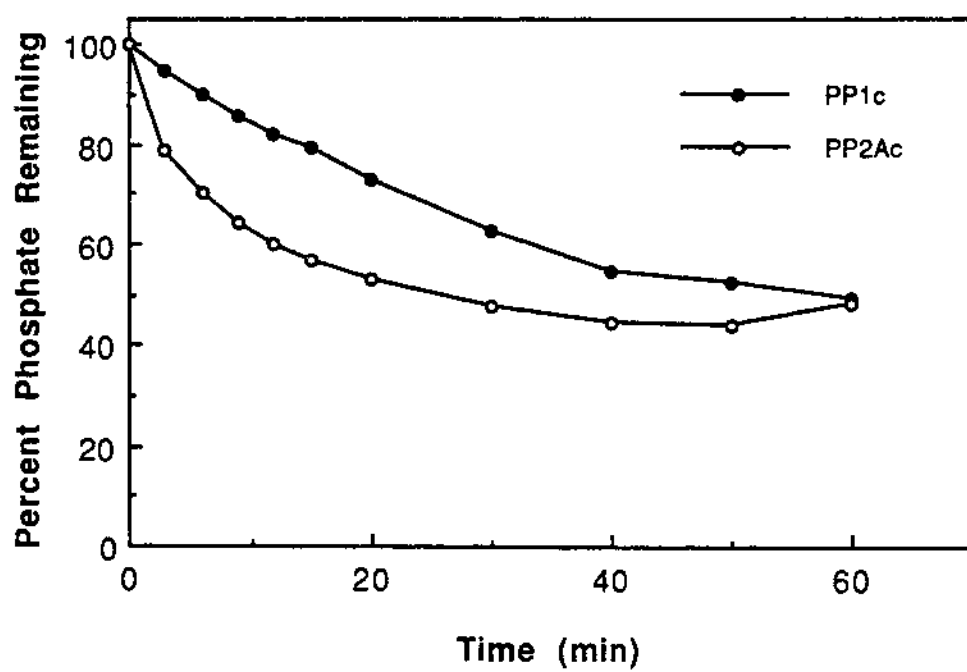


Fig. 21. Double Reciprocal Plot of Initial Velocities vs. PhosphoMLC(3-13) concentration with PP1c. PP1c (1 ng) was incubated with phosphoMLC(3-13) for 6 min at 30°C. Inorganic phosphate liberated was quantitated as described in Methods. PhosphoMLC(3-13) concentration ranged from 2.5-20 μ M.

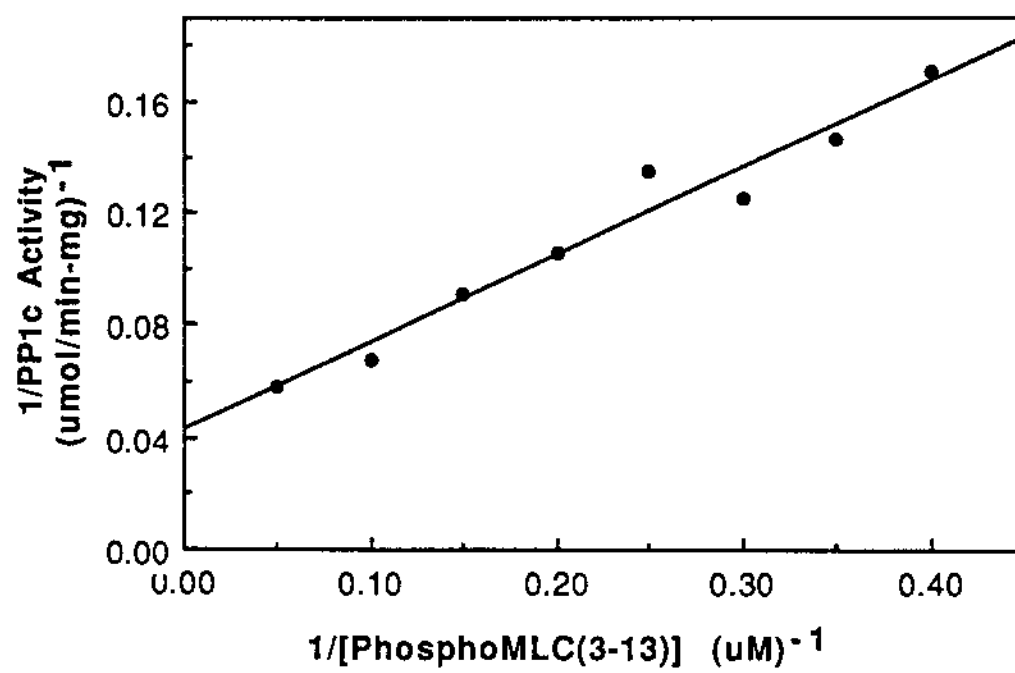


Fig. 22. Double Reciprocal Plot of Initial Velocities vs. PhosphoMLC(3-13) Concentration with PP2Ac. PP2Ac (17 ng) was incubated with phosphoMLC(3-13) for 6 min at 30°C. Inorganic phosphate liberated was quantitated as described in Methods. PhosphoMLC(3-13) concentration ranged from 2.5-20 μ M.

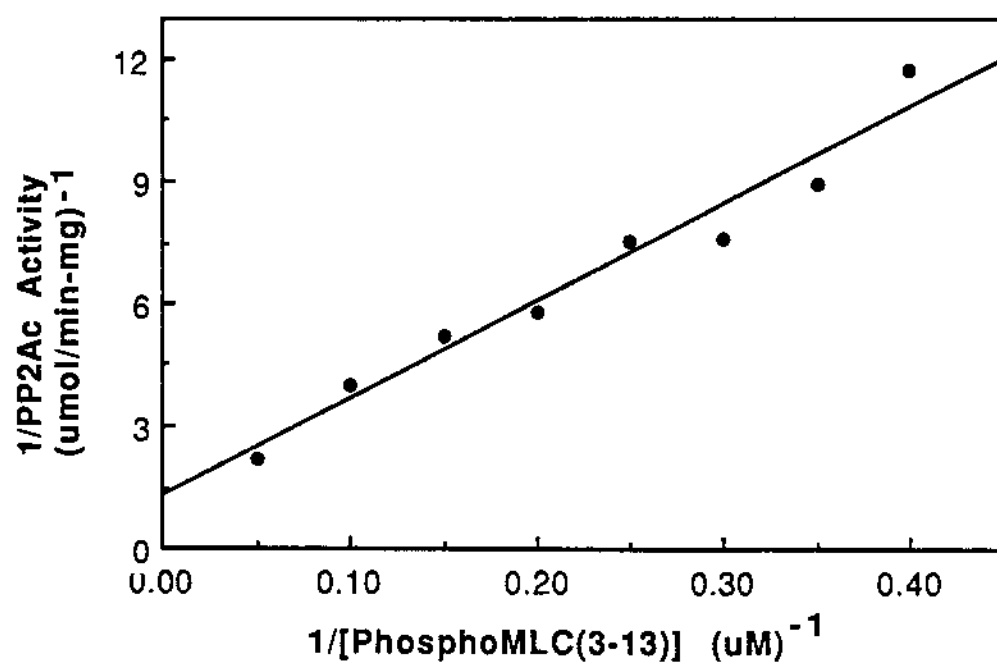


Fig. 23. Double Reciprocal Plot of Initial Velocities vs. PhosphoS6-21 Concentration with PP1c. PP1c (80 ng) was incubated with phosphoS6-21 for 10 min at 30°C. Inorganic phosphate liberated was quantitated as described in Methods. PhosphoS6-21 concentration ranged from 50-250 μ M.

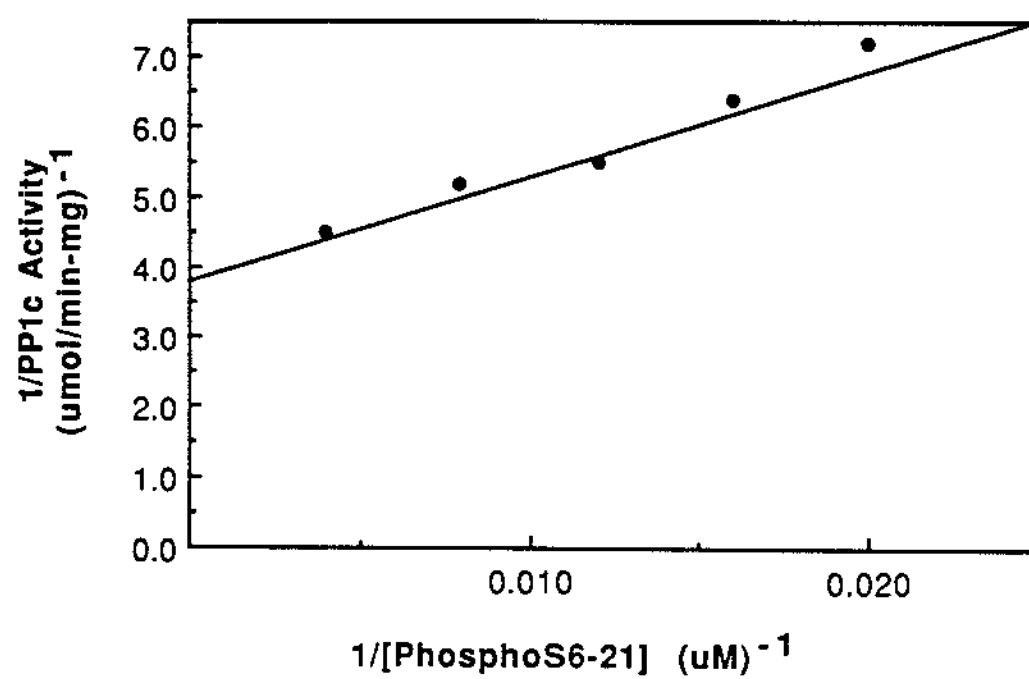


Fig. 24. Double Reciprocal Plot of Initial Velocities vs. PhosphoS6-21 concentration with PP2Ac. PP2Ac (150 ng) was incubated with phosphoS6-21 for 10 min at 30°C. Inorganic phosphate liberated was quantitated as described in Methods. PhosphoS6-21 concentration ranged from 50-250 μ M.

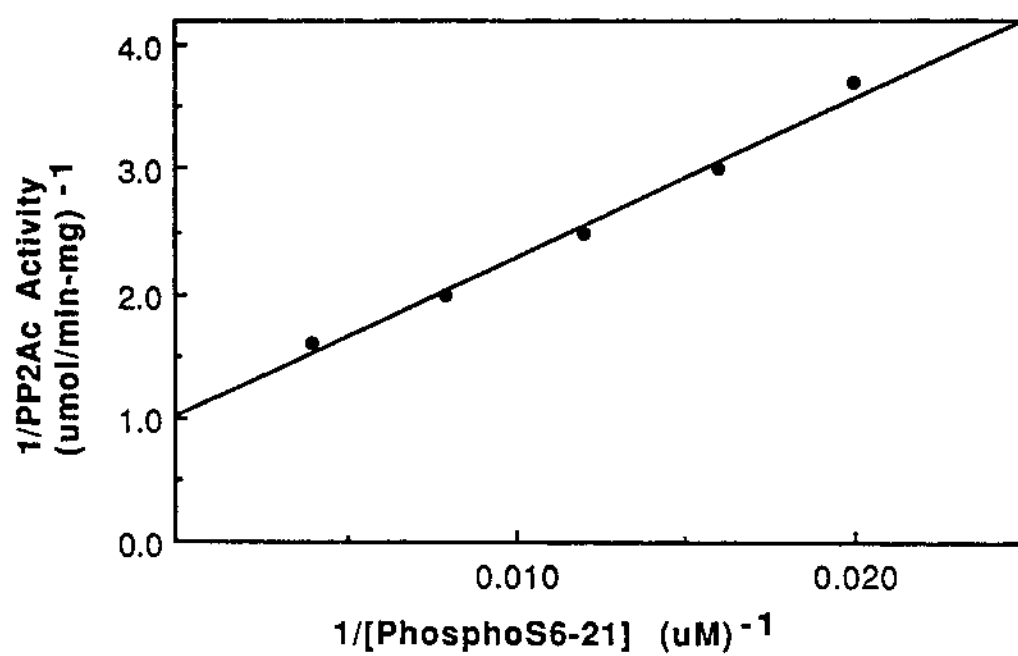


Fig. 25. Effect of Various Phosphatase Inhibitors and Activators on the Dephosphorylation of PhosphoMLC(3-13) by PP1c and PP2Ac.

Various effectors were separately added to the dephosphorylation reaction mixture. PP1c and PP2Ac activities were determined as described in Methods. Concentrations or amounts of effectors used were: MgCl₂ (5 mM); MnCl₂ (5 mM); NaF (50 mM); Na₃VO₄ (50 mM); β-glycerophosphate (50 mM); heparin (50 μg/ml); protamine (25 μg/ml); poly-L-lysine (15 μg/ml); microcystin-LR (1 μM); okadaic acid (1 μM); and I-2 (2 μg/ml).

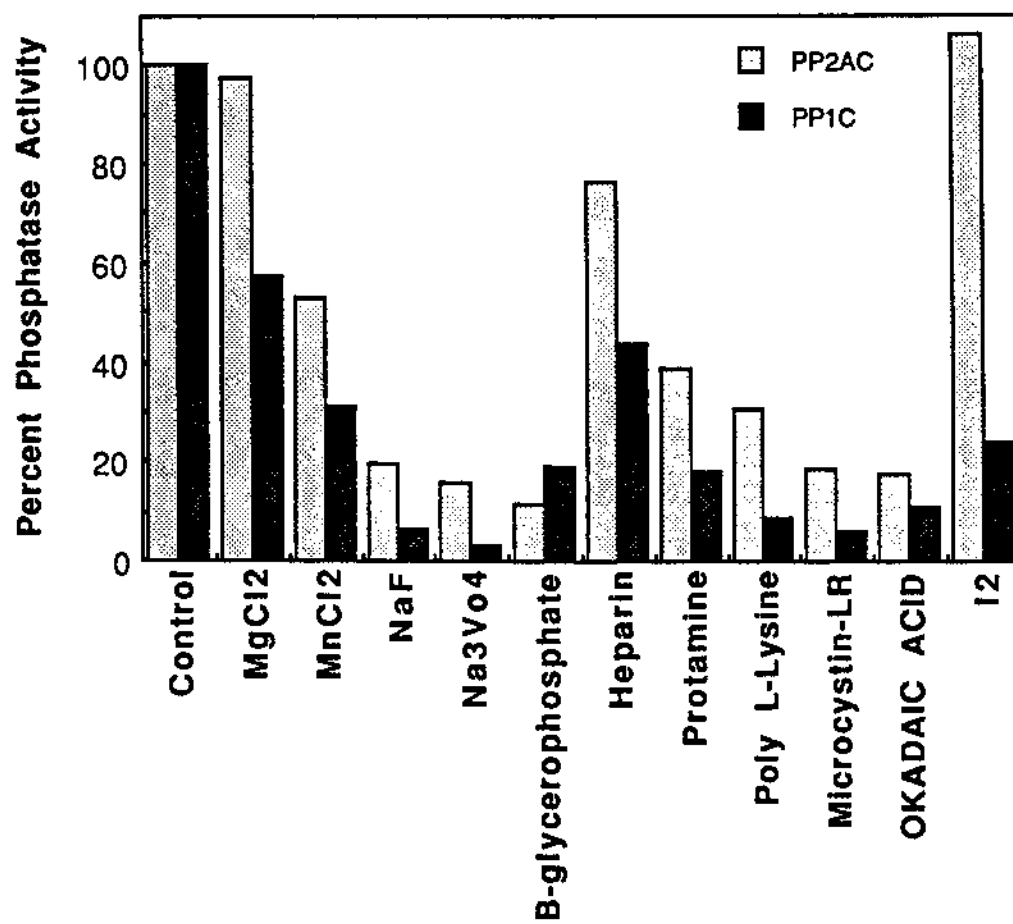


Fig. 26. Effect of Various Phosphatase Inhibitors and Activators on the Dephosphorylation of PhosphoS6-21 by PP1c and PP2Ac.

Various effectors were separately added to the dephosphorylation reaction mixture. PP1c and PP2Ac activities were determined as described in Methods. Concentrations or amounts of effectors used were: MgCl₂ (5 mM); MnCl₂ (5 mM); NaF (50 mM); Na₃VO₄ (50 mM); β -glycerophosphate (50 mM); heparin (50 μ g/ml); protamine (25 μ g/ml); poly-L-lysine (15 μ g/ml); microcystin-LR (1 μ M); okadaic acid (1 μ M); and I-2 (2 μ g/ml).

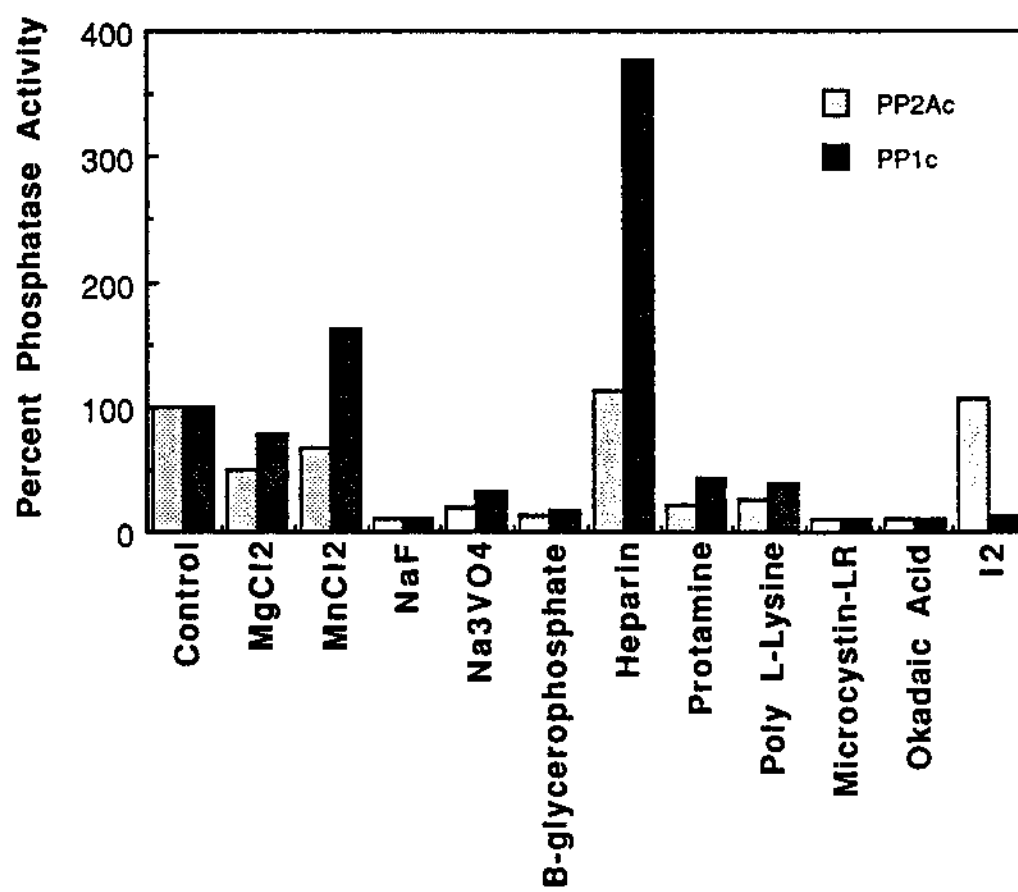


Fig. 27. Dose-Dependent Inhibition of PP1c and PP2Ac by Microcystin-LR. Various concentrations of microcystin-LR were added to the reaction mixture. PP1c and PP2Ac activities with S6-21 were determined as described in Methods. Microcystin-LR concentration ranged from 1.0 pM to 100 μ M.

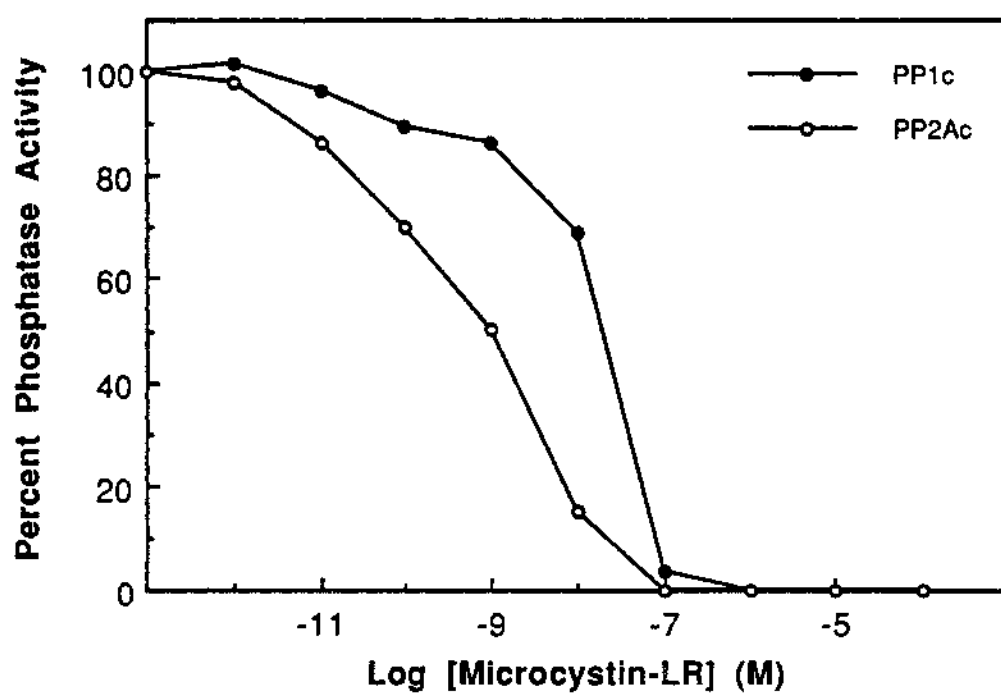
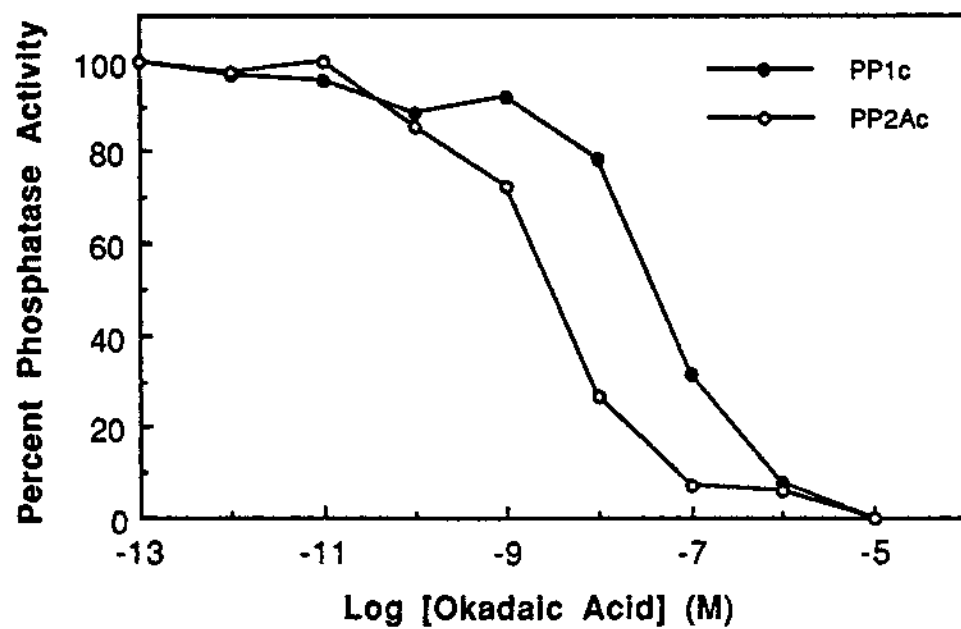


Fig. 28. Dose-Dependent Inhibition of PP1c and PP2Ac by Okadaic Acid. Various concentrations of okadaic acid were added to the reaction mixture. PP1c and PP2Ac activities with S6-21 were determined as described in Methods. Okadaic acid concentration ranged from 1.0 pM to 10 μ M.



CHAPTER IV

DISCUSSION

This study describes the role of substrate in defining the lipid requirements for protein kinase C activation and the role of the primary amino acid sequence in determining the molecular determinants of the substrate specificities of phosphoprotein phosphatases 1 and 2A.

It has been reported that protein kinase C can be isolated from the soluble as well as the particulate fractions of various rat tissues with highest enzyme activity from brain (109). However, human lymphocytes and platelets are excellent sources of protein kinase C with 99% of the enzyme activity mainly associated in the soluble fraction (109). Protein kinase C has been isolated from the soluble fraction of murine lymphosarcoma tumors (196). The specific activity of the enzyme with histone-H1 (8.7 nmol/min-mg) from murine lymphosarcoma tumors is comparable to that reported by Kuo *et al.* (197), but significantly lower than the specific activity (97 nmol/min-mg) of protein kinase C from human lymphocytes and platelets (109). However, the characteristics of protein kinase C from murine lymphosarcoma, in terms of the effector requirements by the enzyme, are quite similar to protein kinase C isolated from other tissues (178, 179).

Protein kinase C isolated from murine lymphosarcoma tumors required the synergistic effect of calcium, phosphatidyl serine, and diacylglycerol to exhibit full activity as determined with both dispersed lipids and lipid micellar

assay. These results are consistent with Hannun *et al.* (178). The enzyme cross reacted with antibodies against protein kinase C- α (Masaracchia, R.A. and Leach, K., personal communication) which is itself a calcium/phospholipid-dependent enzyme suggesting that protein kinase C isolated from murine lymphosarcoma tumors is a protein kinase C- α isoenzyme.

Bazzi and Nelsestuen (127, 128) have reported that protein kinase C substrates play a major role in influencing the effector requirements for the enzyme activation. They demonstrated that protamine phosphorylation by protein kinase C is effector-independent, whereas myelin basic protein phosphorylation by the enzyme requires the presence of phospholipid only. Substrates like histone-3 and myosin light chain require the presence of calcium, phospholipid and diacylglycerol. Hitherto, the molecular determinants behind such selectivity have not been investigated.

In the present study, the presence of the basic residues lysine and arginine from the amino terminus of the peptide substrate did not significantly alter the K_a of OAG required for the activation of protein kinase C. On the other hand, the absence of lysine and arginine from the amino terminus of the peptide substrate did not have any effect on the phosphatidyl serine dependence of protein kinase C activation. With both synthetic peptides MLC(3-13) and MLC(5-13), two molecules of phosphatidyl serine may be sufficient to activate protein kinase C. Hannun *et al.* (178) reported that 6-8 mol% phosphatidyl serine and 2-2.5 mol% diacylglycerol are required for protein kinase C to exhibit full activity when histone-3 was used as the substrate. In addition, results presented in this study indicate that the binding of phosphatidyl serine or OAG

to calcium-protein kinase C is random and not ordered, and the binding of one effector to protein kinase C does not block the binding of the other.

Bazzi and Nelsestuen (127, 128) have indicated that the overall charge of the protein substrate might play a role in determining the effector requirements of protein kinase C activation. Data presented in this study showed that the K_a values of OAG are not significantly different.

The assay procedure described in this study is a rapid, one-step procedure which offers at least the same sensitivity as the existing assays. The procedure is based on the same principles as the popular protein kinase assays employing phosphocellulose papers to separate $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and phosphopeptide (183). The separation of (^{32}P) phosphate from phosphoS6-21 was rapidly and quantitatively accomplished using phosphocellulose papers washed with phosphate buffer. The materials required for this procedure are inexpensive and do not require preequilibration or recycling time. The maximum sensitivity which can be achieved with the assay will depend on the specific activity of the radiolabelled peptide. Peptides which are radiolabelled to a specific activity of 500 dpm/pmol, which is the specific activity of the phosphoS6-21 used in this study, have been routinely prepared in several laboratories (169-172). Additional sensitivity could be achieved by increasing the specific activity of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ used to prepare the modified peptide. The amount of nonspecific background radioactivity even from high specific activity phosphopeptides did not significantly compromise the sensitivity of the assay.

In the experiment shown in Figure 5, PP1c was added to the assay mixture at 10 sec intervals. This time schedule permits the assay of up to 60 samples in one batch if the assay time is 10 min. The washing procedure and

addition of liquid scintillation cocktail for 60 samples requires approximately 30 min. Since it has been established that washing time over 5 min does not effect assay results or background, it is practical to process 60 samples in approximately 1 hr. The most time consuming step in the assay is the liquid scintillation counting.

Dephosphorylation of all phosphopeptides which bind to P81 can be analyzed by this method. The successful application of the P81-based protein kinase assay to a wide variety of synthetic peptides predicts that this phosphatase assay will be feasible with most, if not all, of the synthetic serine/threonine phosphopeptides which have been analyzed to date. The most important criteria for P81 binding will most likely be the net charge of the phosphopeptide in 50 mM NaH_2PO_4 , pH 6.0. As the ratio of basic residues/phosphorylation sites is decreased, it is possible that lower binding affinity of the phosphopeptide on the phosphocellulose may occur. This potential problem may be overcome by lowering the pH of the sodium phosphate wash and thereby titrating both the phosphate residues and acidic amino acids. No significant changes in any assay parameter were observed, including nonspecific background, when 50 mM H_3PO_4 was used as the wash solution. This may be particularly important for the study of casein kinase and tyrosine phosphoprotein phosphatases since these amino acid sequences often contain acidic residues.

The possibility that proteolysis of phosphopeptides may have occurred is not valid, since this assay procedure was utilized to assay for both PP1c and PP2Ac activities in crude preparations and during the purification of both enzymes to homogeneity with no compromise on the sensitivity of the assay. In

addition, dose-dependent inhibition of phosphopeptide dephosphorylation using a wide range of concentrations of the PP1c and PP2Ac potent inhibitors, microcystin-LR and okadaic acid, was consistent in that the amount of (^{32}P)phosphate detected with the P81 paper assay procedure decreased as the phosphatase inhibitor concentration increased.

In summary, many previous studies have shown that phosphopeptide may be quantitated by binding to phosphocellulose papers and this strategy has been used to assay protein kinase activity with numerous synthetic peptides. The P81 binding assay has now been adapted for the quantitation of phosphopeptide dephosphorylation. (^{32}P)Phosphate, a product of the protein phosphatase-catalyzed reaction, is quantitated by pipetting the reaction mixture onto P81 papers and washing the papers with phosphate buffer in a scintillation vial. Unreacted phosphopeptide remains bound to the paper and both the solubilized phosphate and unreacted peptide can be quantitated by liquid scintillation counting. Principal advantages of this method are the large number of samples which can be simultaneously processed and the improved accuracy of the data. In addition, a single analytical strategy, i.e. P81 phosphopeptide binding, can be used for assay of both protein kinase activity and protein phosphatase assay of synthetic peptide substrates.

Phosphoprotein phosphatases 1 and 2A have been purified from a number of tissues (79, 198-200). With phosphorylase *a* the specific activity of PP1 purified from rabbit liver is $4.2\ \mu\text{mol}/\text{min}\cdot\text{mg}$ as reported by Brandt *et al.* (79), whereas that of PP2A isolated from rabbit skeletal muscle is $1.2\ \mu\text{mol}/\text{min}\cdot\text{mg}$ (200). The specific activities of PP1c and PP2Ac isolated from murine lymphosarcoma are 3.8 and $0.51\ \mu\text{mol}/\text{min}\cdot\text{mg}$ respectively, which is

significantly lower than those observed with PP1c and PP2Ac isolated from rabbit skeletal muscle (12-20 and 3 $\mu\text{mol}/\text{min}\cdot\text{mg}$ respectively) (199). These differences in the specific activities of the enzymes from both tissues may be correlated to the functions of those tissues. While lymphoid tissues do not store glycogen, considerable amounts of glycogen particles are present in muscle cells and consequently the two principal glycogen synthase phosphatases PP1 and PP2A would be present in significant quantities. Another reason for such discrepancy is that in this experiment the enzymatic activity is defined as the amount of enzyme that catalyzed the incorporation of 1 μmol of glucose into glycogen per min, and not μmol phosphate liberated from phosphorylase α as presented in the above studies. The specific activity of PP1c purified from lymphosarcoma (presented above) is consistent with that reported for PP1c purified from rat liver with glycogen synthase as a substrate (3.6 μmol glucose incorporated into glycogen/min-mg) as reported by Hiraga *et al.* (198).

Few attempts have been made to apply the synthetic peptide strategy for the determination of substrate specificities of phosphoprotein phosphatases (168-172). In these studies, synthetic peptides were mainly phosphorylated on serine residues by the catalytic subunit of cyclic AMP-dependent protein kinase or phosphorylase kinase on. No other enzymes were used to modify the synthetic peptide substrates. In addition, the apparent K_m values for the dephosphorylation of the synthetic peptides used were relatively high (in the mmolar range) rendering them unsuitable for investigating the substrate specificity of phosphoprotein phosphatases or characterizing these enzymes. In this study, the apparent K_m values for the dephosphorylation of phosphoMLC(3-13) by PP1c and PP2Ac are relatively low (7.1 and 18 μM

respectively). Although higher than obtained for the dephosphorylation of phosphorylase *a* by PP1 (2 μ M) (79), the K_m values presented above for phosphoMLC(3-13) are significantly lower than those obtained with other phosphopeptides studied by other investigators. For example, with the phosphopeptide L-R-R-A-S-V-A-E-L phosphorylated by the catalytic subunit of PKA and dephosphorylated by PP2Ac, the apparent K_m reported by Titanji *et al.* (168) was approximately 28-fold higher than that obtained with phosphoMLC(3-13) in this study. The observed V_{max} was more than 230-fold less than that obtained with phosphoMLC. Overall, these studies indicate that phosphosubstrates phosphorylated by protein kinase C are more favorable substrates for PP1c and PP2Ac than phosphopeptides modified by other kinases such as PKA. In support of this conclusion, phosphoS6-21 phosphorylated by an S6/H4 kinase was a less favorable substrate for PP1c and PP2Ac than protein kinase C-phosphorylated MLC(3-13) as indicated by the kinetic constants of their respective dephosphorylation reactions (Table VII). Further support for this conclusion is that gizzard phospholight chains were not completely dephosphorylated by PP1c and PP2Ac (Fig. 19) as opposed to the synthetic peptide phosphoMLC(3-13) which was completely dephosphorylated by these enzymes (Fig. 17). The reason behind this might be that MLCK phosphorylation site(s) in the light chains which were not dephosphorylated by either PP1c or PP2Ac were unspecifically phosphorylated by protein kinase C.

Although phosphoS6-21 was dephosphorylated by approximately 50% by either phosphatase, HPLC analysis revealed that PP2Ac-dephosphorylation of phosphoS6-21 is site-specific as opposed to PP1c which did not show any specificity. As shown in Figure 14a, peak C, the major phosphopeptide

obtained from the thermolysin digest, was the favored sequence along with peak A (L-S-S) to be dephosphorylated by PP2Ac (Fig. 14b). Other studies in this laboratory (173) have shown that the sequence of the major phosphopeptide (peak C) contain the sequence L-R-A-S-T-S-K-S-E-S-S-Q-K and phosphorylation by the S6/H4 kinase used in this study occurs on one serine (residue 4). As determined by other studies (173) peak D also contains the sequence L-R-A-S-T-S-K- S-E-S-S-Q-K and it is phosphorylated on both serine residues. In this study, peak D was not dephosphorylated by PP2Ac suggesting that multiphosphorylated sequences may be resistant to PP2Ac-catalyzed dephosphorylation. Modification and/or truncation of the phosphopeptide is necessary for the determination of the precise recognition sequence. PP1c was extremely active in dephosphorylating phosphoMLC(3-13) and did not show any selectivity in dephosphorylating phosphoS6-21. Both phosphopeptides contain threonine residue(s), hydrophobic and basic amino acids suggesting that they may be preferred determinants for PP1c-catalyzed dephosphorylation. However, further analyses are necessary before firm conclusions are drawn.

Determining the recognition sequence for phosphoprotein phosphatases is particularly important because such sequence can be a very important tool in determining the role of one specific phosphatase in regulating specific target proteins that possess the recognition sequence of the respective phosphatase.

The effects of various activators and inhibitors on PP1 and PP2A have been reported to be substrate-dependent (104). A number of studies have demonstrated that PP2A is a polycation-activated phosphatase (78, 104). However, Pelech and Cohen (104) have demonstrated that with the right

substrate, such as histone H-1, PP1 can be a polycation-activated enzyme. They demonstrated that basic proteins such as protamine cannot be used to distinguish PP1 from PP2A unless phosphorylase *a* is employed as a substrate. Gergely *et al.* (193) reported that heparin inhibits PP1 when phosphorylase *a* is employed as a substrate. In this study, all effectors shown in Figure 25 inhibited the dephosphorylation of phosphoMLC(3-13) by PP1c and PP2Ac with the exception of $MgCl_2$ which did not have any effect on PP2Ac. Heparin partially inhibited the dephosphorylation of phosphoMLC(3-13) by PP1c and PP2Ac but activated the dephosphorylation of phosphoS6-21 with PP1c by approximately 300%. Both okadaic acid and microcystin-LR completely inhibited the dephosphorylation of phosphoS6-21 by PP1 and PP2Ac, but surprisingly 1 μM of either reagent failed to completely inhibit phosphoMLC(3-13) dephosphorylation. These results are consistent with Pelech and Cohen (104) that the effect of PP1 and PP2A activators and inhibitors may differ depending on the phosphosubstrate utilized. In addition, the I.C. 50 of microcystin-LR and okadaic acid with PP1c and PP2Ac are higher than those observed when phosphorylase *a* is utilized as a substrate. This is in agreement with data from Shenolikar's laboratory (personal communication) which suggests that the degree of inhibition by okadaic acid or microcystin-LR might be substrate dependent as well.

Collectively, these results are consistent with the concept that the effect of various inhibitors and activators on PP1 and PP2A are indeed substrate-dependent. However, the effect of the primary sequences in the phosphosubstrate cannot be concluded from these experiments with the two phosphopeptides phosphoMLC(3-13) and phosphoS6-21, although they are

significantly different from each other. More homologous phosphopeptides could be used in order to compare the effect of individual amino acid residues in the primary sequence of the phosphosubstrate before firm conclusions can be drawn.

In conclusion, this study demonstrates that synthetic peptides can be applied as substrates for reversible phosphorylation. They proved useful in determining the role of primary amino acid sequence in affecting the lipid requirement for protein kinase C activation and in investigating the molecular determinants for the substrate specificity of the catalytic subunits of phosphoprotein phosphatase 1 and 2A.

REFERENCES

1. Hunter, T. (1987) *Cell* **50**, 823-829.
2. Beebe, S.J., and Corbin, J.D. (1986) *The Enzymes* **17**, 44-100.
3. Lincoln, T.M., and Corbin, J.D. (1983) *Adv. Cyclic Nucleotide Res.* **15**, 139-192.
4. Glass, D.B. and Krebs, E.G. (1980) *Annu. Rev. Pharmacol. Toxicol.* **20**, 263-288.
5. Stull, J.T., Nunnally, M.H., and Michinoff, C.H. (1986) *The Enzymes* **17**, 114-159.
6. Nairn, A.C., Hemmings, H.C. Jr, and Greengard, P. (1985) *Annu. Rev. Biochem.* **54**, 931-976.
7. Blackshear, P.J., Nairn, A.C., and Kuo, J.F. (1988) *FASEB J.* **2**, 2957-2969.
8. Rickenberg, H.V., and Leichtling, B.H. (1987) *The Enzymes* **18**, 420-456.
9. Clark, R.B., Kunkel, M.W., Friedman, J., Goka, T.J., and Johnson, J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 1442-1446.
10. Yee, G.H., and Haganir, R.L. (1987) *J. Biol. Chem.* **262**, 6748-6753.
11. Zick, Y., Sagi-Eisenberg, R., Pines, M., Gierschick, P., and Spiegel, A.M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9294-9497.
12. Putney, J.W. Jr. (1988) *J. Exp. Biol.* **139**, 135-150.
13. Nishizuka, Y. (1984) *Nature* **308**, 693-698.
14. Cohen, P. (1982) *Nature* **296**, 613-620.
15. Bell, R.M. (1986) *Cell* **45**, 631-632.
16. Bell, R.M., and Burns, D.J. (1991) *J. Biol. Chem.* **266**, 4661-4664.

17. Nishizuka, Y. (1986) *Science* **233**, 305-312.
18. Nishizuka, Y. (1988) *Nature* **334**, 661-665.
19. Nishizuka, Y. (1989) *Annu. Rev. Biochem.* **58**, 31-44.
20. Hunter, T., and Cooper, J.A. (1985) *Annu. Rev. Biochem.* **54**, 897-930.
21. Gould, K.L., and Nurse, P. (1989) *Nature* **342**, 39-45.
22. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61**, 203-212.
23. Banks, P. (1990) *J. NIH Res.* **2**, 62-66.
24. Gruppuso, P.A., Boylan, J.M., Posner, B.I., Faure, R., and Brautigan, D.L. (1990) *J. Clin. Invest.* **85**, 1754-1760.
25. Roome, J., O'hare, T., Pilch, P.F., and Brautigan, D.L. (1988) *Biochem J.* **256**, 493-500.
26. Tonks, N.K., Diltz, C.D., and Fischer, E.H. (1988) *J. Biol. Chem.* **263**, 6731-6737.
27. Shriner, C.L., and Brautigan, D.L. (1984) *J. Biol. Chem.* **259**, 11383-11390.
28. King, M.J., Sharma, R.P., and Sale, G.J. (1991) *Biochem. J.* **275**, 413-418.
29. Morla, A., Draetta, G., Beach, D., and Wang, J.Y. (1989) *Cell* **58**, 193-203.
30. Tonks, N.K., and Charbonneau, H. (1989) *TIBS* **14**, 497-500.
31. Hunter, T. (1989) *Cell* **58**, 1013-1016.
32. Ingebritsen, T.S., and Cohen, P. (1983) *Science* **221**, 331-338.
33. Meinke, M.H., and Edstrom, R.D. (1991) *J. Biol. Chem.* **266**, 2259-2266.
34. Nimmo, G.A., and Cohen, P. (1978) *Eur. J. Biochem.* **87**, 341-351.
35. Ingebritsen, T.S., Foulkes, J.G., and Cohen, P. (1983) *Eur. J. Biochem.* **132**, 263-274.

36. Ingebritsen, T.S., Blair, J., Guy, P., Witters, L., and Hardie, G. (1983) *Eur. J. Biochem.* **132**, 275-281.
37. Ingebritsen, T.S., Stewart, A.A., and Cohen, P. (1983) *Eur. J. Biochem.* **132**, 297-307.
38. Pato, M.D., Adelstein, R.S., Crouch, D., Safer, B., Ingebritsen, T.S., and Cohen, P. (1983) *Eur. J. Biochem.* **132**, 283-287.
39. Ballou, M., Jenö, P., and Thomas, G. (1988) *J. Biol. Chem.* **263**, 1188-1194.
40. Andres, J.L., Johansen, J.W., and Maller, J.L. (1987) *J. Biol. Chem.* **262**, 14389-14393.
41. Oliver, A.R., and Thomas, G. (1990) *J. Biol. Chem.* **263**, 22460-22466.
42. Andres, J.L., and Maller, J.L. (1989) *J. Biol. Chem.* **264**, 151-156.
43. Novak-Hofer, I., and Thomas, G. (1985) *J. Biol. Chem.* **260**, 10314-10319.
44. Fouikis, J.G., and Maller, J.L. (1982) *FEBS Lett.* **150**, 155-160.
45. Huchon, D., Ozon, R., and Demaille, J.G. (1981) *Nature* **294**, 358-359.
46. Erikson, E., and Maller, J.L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 742-746.
47. Ingebritsen, T.S., and Cohen, P. (1983) *Eur. J. Biochem.* **132**, 255-261.
48. Cohen, P. (1989) *Annu. Rev. Biochem.* **58**, 453-508.
49. Shenolikar, S. (1988) *FASEB J.* **2**, 2753-2764.
50. Holmes, C.F., Luu, H.A., Carrier, F., and Schmitz, F.J. (1990) *FEBS Lett.* **270**, 216-218.
51. Bushfield, M., Lavan, B.E., and Housley, M.D. (1991) *Biochem. J.* **274**, 317-321.

52. Haystead, T.A., Weiel, J.E., Litchfield, D.W., Tsukitani, Y., Fischer, E.H., and Krebs, E.G. (1990) *J. Biol. Chem.* **265**, 16571-16580.
53. Cohen, P., Holmes, C.F., and Tsukitani, Y. (1990) *TIBS* **15**, 98-102.
54. Cohen, P., Klumpp, S., and Schelling, D.L. (1989) *FEBS Lett.* **250**, 596-600.
55. Takai, A., Bialojan, C., Troschka, M., and Rüegg, J.C. (1987) *FEBS Lett.* **217**, 81-84.
56. Bialojan, C., Takai, A., and Rüegg, J.C. (1987) *Adv. Protein Phosphatases* **4**, 253-267.
57. Bialojan, C., and Takai, A. (1988) *Biochem. J.* **256**, 283-290.
58. Honkanon, R.E., Zwiller, J., Moore, R.E., Daily, S.L., Khatra, B.S., Dukelow, M., and Boynton, A.L. (1990) *J. Biol. Chem.* **265**, 19401-19404.
59. MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., and Codd, G.A. (1990) *FEBS Lett.* **2**, 187-192.
60. Eriksson, J.E., Hägerstrand, H., and Isomaa, B. (1987) *Biochim. Biophys. Acta* **930**, 304-310.
61. Cicirelli, M.F. (1992) *Focus* **14**, 16-20.
62. Nishiwaki, S., Fujiki, H., Suganuma, M., Nishiwaki-Matsushima, R., and Sugimura, T. (1991) *FEBS Lett.* **279**, 115-118.
63. Hori, M., Magae, J., Han, Y., Hartshorne, D.J., and Karaki, H. (1991) *FEBS Lett.* **285**, 145-148.
64. Cohen, P.T., Brewis, N.D., Hughes, V., and Mann, D.J. (1990) *FEBS Lett.* **268**, 355-359.
65. Cohen, P., and Cohen, P.T. (1989) *J. Biol. Chem.* **264**, 21435-21438.

66. Ohkura, H., Kinoshita, N., Migatani, S., Toda, T., and Yanagida, M. (1989) *Cell* **57**, 997-1007.
67. Stone, S.R., Hofsteenge, J., and Hemmings, B.A. (1987) *Biochemistry* **26**, 7215-7220.
68. Kincaid, R.L., Nightingale, M.S., and Martin, B.M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8983-8987.
69. MacGowan, C.H., Campbell, D.G., and Cohen, P. (1989) *Biochim. Biophys. Acta* **930**, 279-282.
70. MacGowan, C.H., and Cohen, P. (1987) *Eur. J. Biochem.* **166**, 713-722.
71. Cohen, P.T. (1988) *FEBS Lett.* **232**, 17-23.
72. Bai, G., Zhang, Z., Amin, J., Deans-Zirattu, S.A., and Lee, E.Y. (1988) *FASEB J.* **2**, 3010-3016.
73. Dombrádi, V., Axton, J.M., Glover, D.M., and Cohen, P.T. (1989) *Eur. J. Biochem.* **183**, 603-610.
74. Booher, R., and Beach, D. (1989) *Cell* **57**, 1009-1016.
75. Doonan, J.H., and Morris, N.R. (1989) *Cell* **57**, 987-996.
76. Cohen, P.T., and Dombrádi, V. (1989) *Adv. Protein Phosphatases* **5**, 447-463.
77. MacKintosh, C., Coggins, J.R., and Cohen, P. (1989) *Biochem. J.* **262**, 335-339.
78. Cornwell, T., Mehta, P., and Shenolikar, S. (1987) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**, 373-382.
79. Brandt, H., Capulong, Z., and Lee, E.Y. (1975) *J. Biol. Chem.* **250**, 8083-8044.

80. Brandt, H., Killilea, D., and Lee, E.Y. (1974) *Biochem. Biophys. Res. Comm.* **61**, 598-604.
81. Jakes, S., and Schlender, K.K. (1988) *Biochim. Biophys. Acta* **975**, 11-16.
82. Stralfors, P., Hiraga, A., and Cohen, P. (1985) *Eur. J. Biochem.* **149**, 295.
83. Hiraga, A., Kemp, B.E., and Cohen, P. (1987) *Eur. J. Biochem.* **163**, 253.
84. Hubbard, M.J., and Cohen, P. (1989) *Eur. J. Biochem.* **180**, 457-465.
85. Stalmans, W., Bollen, M., and Mvumbi, L. (1987) *Diabetes Metab. Rev.* **3**, 127-161.
86. MacKintosh, C., Kampbell, D.G, Hiraga, A., and Cohen, P. (1988) *FEBS Lett.* **234**, 189-194.
87. Li, H., Price, D.J., and Tabarini, D. (1985) *J. Biol. Chem.* **260**, 6416-6426.
88. Abeele, C.V., Vandenheede, J.R., and Merlevede, W. (1987) *J. Biol. Chem.* **262**, 14086-14089.
89. Cohen, P. (1988) *Mol. Aspects Cell. Regul.* **5**, 123-144.
90. Dent, P., Lavoinnie, A., Nakielnny, S., Caudwell, B., Watt, P., and Cohen, P. (1990) *Nature* **348**, 302-308.
91. Hubbard, M., and Cohen, P. (1989) *Eur. J. Biochem.* **186**, 701-709.
92. Klee, C.B., and Cohen, P. (1988) *Mol. Aspects Cell. Regul.* **5**, 225-248.
93. Chisholm, A.A., and Cohen, P. (1988) *Biochim. Biophys. Acta* **968**, 392-400.
94. Chisholm, A.A., and Cohen, P. (1988) *Biochim. Biophys. Acta* **971**, 163-169.
95. Pato, M.D., and Kere, E. (1985) *J. Biol. Chem.* **260**, 12359-12366.
96. Price, D.J., and Li, H.C. (1985) *Biochem. Biophys. Res. Comm.* **128**, 1203-1210.

97. Foulkes, J.G., Ernst, V., and Levin, D.H. (1983) *J. Biol. Chem.* **258**, 1439-1443.
98. Olivier, A.R., Ballou, L.M., and Thomas, G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4720-4724.
99. Jenö, P., Ballou, L.M., Novak-Hofer, I., and Thomas, G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 406-410.
100. Sturgill, T.W., Ray, L.B., Erikson, E., and Maller, J.L. (1988) *Nature* **334**, 715-718.
101. Pain, V.M. (1986) *Biochem. J.* **235**, 625-637.
102. Ernst, V., Levin, D.H., Foulkes, J.G., and London, I.M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7092-7096.
103. Petryshyn, R., Levin, D.H., Foulkes, J.G., and London, I.M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6512-6516.
104. Pelech, S., and Cohen, P. (1985) *Eur. J. Biochem.* **148**, 245-251.
105. Goris, J., Waelkens, E., and Merlevede, W. (1985) *FEBS Lett.* **188**, 262-266.
106. Goris, J., Waelkens, E., and Merlevede, W. (1986) *Biochem. J.* **239**, 109-114.
107. Nishizuka, Y. (1984) *Science* **225**, 1365-1370.
108. Berridge, M.J. (1984) *Biochem. J.* **220**, 345-360.
109. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 13341-13348.
110. Wise, B.C., Raynor, R.L., and Kuo, J.F. (1982) *J. Biol. Chem.* **257**, 8481-8488.
111. Bishop, W., and Bell, R.M. (1988) *Oncogene Res.* **2**, 205-218.

112. Huang, K-P. (1989) *Trends Neurosci.* **12**, 425-432.
113. Parker, P.J., Kour, G., Marais, R., Mitchell, F., Pears, C., Schaap, D., Stabel, S., and Webster, C. (1989) *Mol. Cell. Endocrinol.* **65**, 1-11.
- 114.
115. Berridge, M.J. (1987) *Ann. Rev. Biochem.* **56**, 159.
116. Exton, J.H. (1990) *J. Biol. Chem.* **265**, 1-4.
117. Batty, I.R., Nahorski, S.R., and Irvine, R.F. (1985) *Biochem. J.* **232**, 211-215.
118. Palmer, S., Hawkins, P.T., Mitchell, R.H., and Kirk, C.J. (1986) *Biochem. J.* **238**, 491-499.
119. Irvine, R.F. Letcher, A.J., Lander, D.J., and Berridge, M.J. (1986) *Biochem. J.* **240**, 301-304.
120. Nishikawa, M., Hidaka, H., and Adelstein, R.S. (1983) *J. Biol. Chem.* **258**, 14069-14072.
121. Naka, M., Nishikawa, M., Adelstein, R.S., and Hidaka, H. (1983) *Nature (Lond.)* **306**, 490-492.
122. Nishikawa, M., Sellers, J.R., Adelstein, R.S., and Hidaka, H. (1984) *J. Biol. Chem.* **259**, 8808-8814.
123. LePeuch, D.J., Ballester, R., and Rosen, O.M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6858-6862.
124. Ahmed, Z., Lee, F.T., DePaoli-Roach, A., and Roach, P.J. (1984) *J. Biol. Chem.* **259**, 8743-8747.
125. Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7610-7616.
126. Hunter, T., Ling, N., and Cooper, J.A. (1984) *Nature* **311**, 480-483.

127. Bazzi, M.D., and Nelsestuen, G.L. (1987) *Biochemistry* **26**, 1974-1982.
128. Bazzi, M.D., and Nelsestuen, G.L. (1987) *Biochemistry* **26**, 5002-5008.
129. Parker, P., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M., and Ullrich, A. (1986) *Science* **233**, 853-859.
130. Coussens, L., Parker, P., Rhee, L., Yang-Feng, T., Chen, E., Waterfield, M., Franke, U., and Ullrich, A. (1986) *Science* **233**, 859-866.
131. Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K., and Nishizuka, Y. (1986) *FEBS Lett.* **206**, 347-352.
132. Knopf, J., Lee, M-H., Sultzaman, L., Kriz, R., Loomis, C., Hedwick, R., and Bell, R.M. (1986) *Cell* **46**, 491-502.
133. Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., and Hidaka, H. (1987) *Nature* **325**, 161-166.
134. Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asoaka, Y., Sekiguchi, K., Ase, K., Igarashi, K., and Nishizuka, Y. (1987) *Science* **236**, 1116-1120.
135. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1987) *FEBS Lett.* **226**, 125-128.
136. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1988) *J. Biol. Chem.* **263**, 6927-6943.
137. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) *Cell* **53**, 731-741.
138. Batcher, N., Zisman, Y., Berenet, T., and Livneh, E. (1991) *Mol. Cell. Biol.* **11**, 126-133.
139. Osada, S., Mizuno, K., Saido, T.C., Akita, Y., Suzuki, K., Kurok, T., and Ohno, S. (1990) *J. Biol. Chem.* **265**, 22434-22440.

140. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3099-3103.
141. Farago, A., and Nishizuka, Y. (1990) *FEBS Lett.* **268**, 350-354.
142. Klug, A., and Rhodes, D. (1987) *TIBS* **12**, 464-469.
143. Yoshida, Y., Huang, F.L., Nakabayashi, H., and Huang, K. (1988) *J. Biol. Chem.* **263**, 9868-9873.
144. Akita, Y., Ohno, S., Yasuhiko, K., Yano, A., and Suzuki, K. (1990) *J. Biol. Chem.* **265**, 354-362.
145. Kiley, S., Parker, P.J., Fabbro, D., and Jaken, S. (1991) *J. Biol. Chem.* **266**, 23761-23768.
146. Kemp, B.E., Benjamini, E., and Krebs, E.G. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1038-1042.
147. House, C., Wettenhall, R.E., and Kemp, B.E. (1987) *J. Biol. Chem.* **262**, 772-777.
148. Hassel, T.C., Magnino, P.E., and Masaracchia, R.A. (1989) *Biochem. Biophys. Acta* **957**, 1-10.
149. Gabrielli, B., Wettenhall, R.E. Kemp, B.E., Quinn, M., and Bizonova, L. (1984) *FEBS Lett.* **175**, 219-226.
150. Dennis, P.B., Brandon, S.D., and Masaracchia, R.A. (1990) *Biochem. Biophys. Res. Comm.* **173**, 673-679.
151. Michnoff, C.H., Kemp, B.E., and Stull, J.T. (1986) *J. Biol. Chem.* **261**, 8320-8326.
152. Colbran, R.J., Fong, Y., Schworer, C.M., and Soderling, T.R. (1988) *J. Biol. Chem.* **263**, 18145-18151.
153. Glass, D.B., and Krebs, E.G. (1979) *J. Biol. Chem.* **254**, 9728-9738.

154. Kemp, B.E., Graves, D.J., Benjamini, E., and Krebs, E.G. (1977) *J. Biol. Chem.* **252**, 4888-4894.
155. Kemp, B.E., Pearson, R.B., and House, C. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7471-7475.
156. Pearson, R.B., Jakes, R., John, M., Kendrick-Jones, J., and Kemp, B.E. (1984) *FEBS Lett.* **168**, 108-112.
157. Kemp, B.E., and Pearson, R.B. (1985) *J. Biol. Chem.* **260**, 3355-3359.
158. Kemp, B.E., Pearson, R.B., and House, C. (1982) *J. Biol. Chem.* **257**, 13349-13353.
159. Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1985) *J. Biol. Chem.* **260**, 12492-12499.
160. Woodgett, J., Gould, K., and Hunter, T. (1986) *Eur. J. Biochem.* **161**, 177-184.
161. O'brian, C.A., Lawrence, D.S., Kaiser, E.T., and Weinstein, B. (1984) *Biochem. Biophys. Res. Comm.* **124**, 296-302.
162. Bengur, A.R., Robinson, E.A., Appella, E., and Sellers, J. (1987) *J. Biol. Chem.* **262**, 7613-7617.
163. Ikebe, M., Harshorne, D.J., and Elzinga, M. (1987) *J. Biol. Chem.* **262**, 9569-9573.
164. Ferrari, S., Marchiori, F., Borin, G., and Pinna, L.A. (1988) *FEBS Lett.* **184**, 72-77.
165. Turner, R.S., Kemp, B.E., de Su, H., and Kuo, J.F. (1985) *J. Biol. Chem.* **260**, 11503-11507.

166. Eckols, T.K., Thompson, R.E., and Masaracchia, R.A. (1983) *Eur. J. Biochem.* **134**, 249.
167. Kemp, B., and Pearson, R. (1990) *TIBS* **15**, 342-346.
168. Titanji, V.P., Ragnarsson, U., Humble, E., and Zetterqvist, Ö. (1980) *J. Biol. Chem.* **255**, 11339-11343.
169. Deana, A.D., MacGowan, C.H., Cohen, P., Marchiori, F., Meyer, H.E., and Pinna, L.A. (1990) *Biochim. Biophys. Acta* **1051**, 199-202.
170. McNall, S.J., and Fischer, E.H. (1988) *J. Biol. Chem.* **263**, 1893-1897.
171. Agostinis, P., Goris, J., Waelkens, E., Pinna, L.A., Marchiori, F., and Merlevede, W. (1987) *J. Biol. Chem.* **262**, 1060-1064.
172. Agostinis, P., Goris, J., Pinna, L.A., Marchiori, F., perich, J.W., Meyer, H.E., and Merlevede, W. (1990) *Eur. J. Biochem.* **198**, 235-241.
173. Dennis, P., Brandon, S.D., and Masaracchia, R.A. (1990) *Biochem. Biophys. Res. Comm.* **173**, 673-679.
174. Brandon, S.D., and Masaracchia, R.A. (1991) *J. Biol. Chem.* **266**, 380-385.
175. DeGuzman, A., and Lee, E.Y. (1988) *Meth. Enzymol.* **159**, 356-368.
176. Merrifield, R.B. (1964) *Biochemistry* **3**, 1385-1390.
177. Hodges, R.S., and Merrifield, R.B. (1975) *Anal. Biochem.* **65**, 241-272.
178. Hannun, Y.A., Loomis, C.R., and Bell, R.M. (1985) *J. Biol. Chem.* **260**, 10039-10043.
179. Hannun, Y.A., and Bell, R.M. (1986) *J. Biol. Chem.* **261**, 9341-9347.
180. Hassell, T.C., Kemp, B., and Masaracchia, R.A. (1988) *Biochem. Biophys. Res. Comm.* **134**, 240-247.
181. Westall, F., and Hesser, W. (1974) *Anal. Biochem.* **61**, 610-613.

182. Roth, M. (1971) *Anal. Chem.* **43**, 880-882.
183. Glass, D.B., Masaracchia, R.A., Feramisco, J.R., and Kemp, B.E. (1978) *Anal. Biochem.* **87**, 566-575.
184. Gilboe, D.P., Larson, K.L., and Nuttall, F.Q. (1972) *Anal. Biochem.* **47**, 20-27.
185. Bradford, M.A. (1976) *Anal. Biochem.* **72**, 248-259.
186. Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701-6704.
187. Lapetina, E.G., Reep, B., Ganong, B.R., and Bell, R.M. (1985) *J. Biol. Chem.* **260**, 1358-1361.
188. Ebeling, J.G., Vandebark, G.R., Kuhn, L., Ganong, B., Bell, R., and Niedel, J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 815-819.
189. Jetten, A.M., Ganong, B.R., Vandebark, G.R., Shirley, J.E., and Bell, R.M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1941-1945.
190. Davis, R.J., Ganong, B.R., Bell, R.M., and Czech, M.P. (1985) *J. Biol. Chem.* **260**, 1562-1566.
191. Niedel, J.E., Kuhn, L., and Vandebark, G.R. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 36-40.
192. Foulkes, J.G., Strada, S.J., Henderson, P.J., and Cohen, P. (1983) *Eur. J. Biochem.* **132**, 309-313.
193. Gergely, P., Erdöd, F., and Bot, G. (1984) *FEBS Lett.* **169**, 45-48.
194. Erdöd, F., Csontos, C., Bot, G., and Gergely, P. (1985) *Biochim. Biophys. Acta* **827**, 23-29.
195. Krivanek, J., and Novakova, L. (1991) *FEBS Lett.* **282**, 32-34.
196. Magnino, P. (1984) Master's Thesis, U. of North Texas.

197. Kuo, J.F., Anderson, R.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M., and Wrenn, R.W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7039-7043.
198. Hiraga, A., Kikuchi, K., Tamura, S., and Tsuiki, S. (1981) *Eur. J. Biochem.* **119**, 503-510.
199. Tung, Y.M., Resink, T.J., Hemmings, B.A., Shenolikar, S., and Cohen, P. (1984) *Eur. J. Biochem.* **138**, 635-641.
200. Waelkens, E., Goris, J., and Merlevede, W. (1987) *J. Biol. Chem.* **262**, 1049-1059.