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Immunological and Substrate Specificity Characterization of a Novel cdc2-like Kinase

by

Katherine N. Beaudette

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Anthropology
- Archaeology 0324
- Cultural 0326
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- General 0310
- Accounting 0272
- Banking 0770
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Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES
Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

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Biogeochemistry 0425
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Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
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Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
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Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

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Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences
Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY
General 0621
Behavioral 0384
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Developmental 0620
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Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
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Art 0273
Collèges communautaires 0275
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Enseignement primaire 0524
Enseignement professionnel 0747
Enseignement religieux 0527
Enseignement secondaire 0533
Enseignement spécial 0529
Enseignement supérieur 0745
Évaluation 0288
Finances 0277
Formation des enseignants 0530
Histoire de l'éducation 0520
Langues et littérature 0279
Lecture 0535
Mathématiques 0280
Musique 0522
Orientation et consultation 0519
Philosophie de l'éducation 0998
Physique 0523
Programmes d'études et enseignement 0727
Psychologie 0525
Sciences 0714
Sciences sociales 0534
Sociologie de l'éducation 0340
Technologie 0710

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Langues
- Généralités 0679
- Anciennes 0289
- Linguistique 0290
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- Généralités 0401
- Anciennes 0294
- Comparée 0295
- Médiévale 0297
- Moderne 0298
- Africaine 0316
- Américaine 0591
- Anglaise 0593
- Asiatique 0305
- Canadienne (Anglaise) 0352
- Canadienne (Française) 0355
- Germanique 0311
- Latino-américaine 0312
- Moyen-orientale 0315
- Romane 0313
- Slave et est-européenne 0314

PHILOSOPHIE, RELIGION ET THÉOLOGIE
Philosophie 0422
Religion
- Généralités 0318
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- Études bibliques 0321
- Histoire des religions 0320
- Philosophie de la religion 0322

Théologie 0469

SCIENCES SOCIALES
Anthropologie
- Archéologie 0324
- Culturelle 0326
- Physique 0327

Droit 0398
Économie
- Généralités 0501
- Commerce-Affaires 0505
- Économie agricole 0503
- Économie du travail 0510
- Finances 0508
- Histoire 0509
- Théorie 0511

Études américaines 0323
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Folklore 0358
Géographie 0366
Gérontologie 0351
Gestion des affaires
- Généralités 0310
- Administration 0454
- Banques 0770
- Comptabilité 0272
- Marketing 0338

Histoire
- Histoire générale 0578
- Ancienne 0579
- Médiévale 0581
- Moderne 0582
- Histoire des noirs 0328
- Africaine 0331
- Canadienne 0334
- États-Unis 0337
- Européenne 0335
- Moyen-orientale 0333
- Latino-américaine 0336
- Asie, Australie et Océanie 0332

Histoire des sciences 0585
Loisirs 0814
Planification urbaine et régionale 0999
Science politique
- Généralités 0615
- Administration publique 0617
- Droit et relations internationales 0616

Sociologie
- Généralités 0626
- Aide et bien-être social 0630
- Criminologie et établissements pénitentiaires 0627
- Démographie 0938
- Études de l' individu et de la famille 0628
- Études des relations interethniques et des relations raciales 0631
- Structure et développement social 0700
- Théorie et méthodes 0344
- Travail et relations industrielles 0629

Transports 0709
Travail social 0452

SCIENCES ET INGÉNIERIE

SCIENCES BIOLOGIQUES
Agriculture
- Généralités 0473
- Agronomie 0285
- Alimentation et technologie alimentaire 0359
- Culture 0479
- Élevage et alimentation 0475
- Exploitation des pâturages 0777
- Pathologie animale 0476
- Pathologie végétale 0480
- Physiologie végétale 0817
- Sylviculture et faune 0478
- Technologie du bois 0746

Biologie
- Généralités 0306
- Anatomie 0287
- Biologie (Statistiques) 0308
- Biologie moléculaire 0307
- Botanique 0309
- Cellule 0379
- Écologie 0329
- Entomologie 0353
- Génétique 0369
- Limnologie 0793
- Microbiologie 0410
- Neurologie 0317
- Océanographie 0416
- Physiologie 0433
- Radiation 0821
- Science vétérinaire 0778
- Zoologie 0472

Biophysique
- Généralités 0786
- Medicale 0760

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Biogéochimie 0425
Géochimie 0996
Géodésie 0370
Géographie physique 0368
Géologie 0372
Géophysique 0373
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Minéralogie 0411
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Paléoécologie 0426
Paléontologie 0418
Paléozoologie 0985
Palynologie 0427

SCIENCES DE LA SANTÉ ET DE L'ENVIRONNEMENT
Économie domestique 0386
Sciences de l'environnement 0768
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- Généralités 0566
- Administration des hôpitaux 0769
- Alimentation et nutrition 0570
- Audiologie 0300
- Chimiothérapie 0992
- Dentisterie 0567
- Développement humain 0758
- Enseignement 0350
- Immunologie 0982
- Loisirs 0575
- Médecine du travail et thérapie 0354
- Médecine et chirurgie 0564
- Obstétrique et gynécologie 0380
- Ophtalmologie 0381
- Orthophonie 0460
- Pathologie 0571
- Pharmacie 0572
- Pharmacologie 0419
- Physiothérapie 0382
- Radiologie 0574
- Santé mentale 0347
- Santé publique 0573
- Soins infirmiers 0569
- Toxicologie 0383

SCIENCES PHYSIQUES
Sciences Pures
Chimie
- Généralités 0485
- Biochimie 487
- Chimie agricole 0749
- Chimie analytique 0486
- Chimie minérale 0488
- Chimie nucléaire 0738
- Chimie organique 0490
- Chimie pharmaceutique 0491
- Physique 0494
- PolymÇres 0495
- Radiation 0754

Mathématiques 0405
Physique
- Généralités 0605
- Acoustique 0986
- Astronomie et astrophysique 0606
- Electronique et électricité 0607
- Fluides et plasma 0759
- Météorologie 0608
- Optique 0752
- Particules (Physique nucléaire) 0798
- Physique atomique 0748
- Physique de l'état solide 0611
- Physique moléculaire 0609
- Physique nucléaire 0610
- Radiation 0756

Statistiques 0463

Sciences Appliqués Et Technologie
Informatique 0984
Ingénierie
- Généralités 0537
- Agricole 0539
- Automobile 0540
- Biomédicale 0541
- Chaleur et ther modynamique 0348
- Conditionnement (Emballage) 0549
- Génie aérospatial 0538
- Génie chimique 0542
- Génie civil 0543
- Génie électronique et électrique 0544
- Génie industriel 0546
- Génie mécanique 0548
- Génie nucléaire 0552
- Ingénierie des systèmes 0790
- Mécanique navale 0547
- Métallurgie 0743
- Science des matériaux 0794
- Technique du pétrole 0765
- Technique minière 0551
- Techniques sanitaires et municipales 0554
- Technologie hydraulique 0545

Mécanique appliquée 0346
Géotechnologie 0428
Matières plastiques (Technologie) 0795
Recherche opérationnelle 0796
Textiles et tissus (Technologie) 0794

PSYCHOLOGIE
Généralités 0621
Personnalité 0625
Psychobiologie 0349
Psychologie clinique 0622
Psychologie du comportement 0384
Psychologie du développement 0620
Psychologie expérimentale 0623
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Psychologie sociale 0451
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Immunological and Substrate Specificity Characterization of a Novel cdc2-like Kinase" submitted by Katherine N. Beaudette in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

A proline-directed protein kinase (PDK) from bovine brain has been purified (Lew *et al.*, 1992a) and found to consist of a 33kDa catalytic subunit having high sequence homology to $p34^{cdc2}$ and cdk2 ((Lew *et al.*, 1992b). Substrate specificity determinants for this enzyme were examined using synthetic peptide substrates derived from the *in vitro* $p34^{cdc2}$ phosphorylation sites of histone H1. The peptide P-K-T-P-K-K-A-K-K-L was found to be an excellent substrate for the brain PDK, having a K_m value in the micromolar range. Important determinants for efficient substrate phosphorylation in this peptide were found both within the proposed substrate consensus motif (**S/T**-P-X-K/R) of $p34^{cdc2}$ kinase and outside of this sequence. In addition to the absolute requirement for a proline residue immediately C-terminal to the phosphorylatable residue (+1) and a basic residue at the +3 position, a basic amino acid at the +2 position was greatly preferred over an acidic amino acid. A proline residue at the -2 position and a cluster of basic amino acids further C-terminal to the consensus motif were also found to be important for substrate binding. The HeLa cell $p34^{cdc2}$ kinase displays similar specificity to that of the brain PDK, as the additional determinants outside of the consensus motif that contribute to the efficient phosphorylation of the histone peptides by the brain PDK, also appear to be important for $p34^{cdc2}$ catalyzed phosphorylation.

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DEDICATION

For Lee

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
DEAE	diethyl amino ethyl
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol bis(β-aminoethyl ether)N-N-N'-N' tetraacetic acid
Fmoc	9-fluorenylmethoxycarbonyl
HEPES	4-(2-hydroxyethyl)-1-piperazinemethane-sulphonic acid
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
KLH	keyhole limpet hemocyanin
MLCK	myosin light chain kinase
MOPS	4-morpholinepropanesulfonic acid
NBT	p-nitro blue tetrazolium chloride
NP-40	nonidet 40
PAGE	polyacrylamide gel electrophoresis
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
Tris	tris(hydroxymethylamino) methane

INTRODUCTION

At the time $p34^{cdc2}$ was discovered as a key serine/threonine kinase regulating the cell cycle in yeast, no one could have predicted the implications of its discovery on the study of cellular proliferation and regulation in higher order species. In both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the function of this gene product is required both for the entry into mitosis and for the traversal of the G_1 control point Start, in which the cell becomes committed to beginning S phase (Beach *et al.*, 1982, Nurse and Bisset, 1981). The genetic approaches used to study the yeast cell cycle became linked to the biochemical approaches used by cell biologists when $p34^{cdc2}$ was identified as a component of maturation promoting factor or MPF, a factor that was able to induce Xenopus oocytes naturally arrested at meiosis I to undergo repeated cell cycles (Masui and Markert, 1971, Reynhout and Smith, 1974). Using a cell free assay system (Lohka and Maller, 1985, Lohka and Masui, 1983), in which many of the processes of mitosis occurred, MPF was purified to homogeneity and shown to contain a Xenopus homolog of the yeast $p34^{cdc2}$ (Dunphy *et al.*, 1988, Gautier *et al.*, 1988). At around the same time, various groups also identified $p34^{cdc2}$ as a component of the M-phase specific histone H1 kinase or growth associated kinase, a protein kinase that is transiently activated in a wide variety of dividing cells at the G_2/M transition (Arion *et al.*, 1988, Labbe *et al.*, 1989, Labbe *et al.*, 1988). The discovery of $p34^{cdc2}$ as a component of MPF and the identification of functional homologues in a wide variety of species including humans (Lee and Nurse, 1987), led to the proposal that $p34^{cdc2}$ was a universal regulator of the cell cycle (Nurse, 1990).

Regulation of $p34^{cdc2}$ Activity

The regulation of $p34^{cdc2}$ has been intensely studied and to date its activity is known to be controlled not by variations in protein levels (Draetta and Beach, 1988,

Durkacz *et al.*, 1986, Simanis and Nurse, 1986), but by its physical associations with other proteins and by cell cycle dependent phosphorylation events (Draetta, 1990, Nurse, 1990 for review).

Prominent partners of $p34^{cdc2}$ are the cyclins (Pines, 1991, Pines and Hunter, 1991a), proteins first identified in sea urchin and clams because of their periodic accumulation and degradation within the cell cycle (Evans *et al.*, 1983, Swenson *et al.*, 1986). In addition to the A and B-type cyclins, the cyclin family has grown to include cyclins C, D1 also known as PRAD1 or CYL1, D2, D3 and E (Koff *et al.*, 1991, Leopold and O'Farrell, 1991, Lew *et al.*, 1991, Matsushime *et al.*, 1991, Motokura *et al.*, 1991, Pines and Hunter, 1989, Xiong *et al.*, 1991) as well as the CLN cyclins identified in yeast (Hadwiger *et al.*, 1989, Nash *et al.*, 1988). Select members of this family are known to associate with $p34^{cdc2}$ or closely related homologues of $p34^{cdc2}$. The best characterized interaction is that of $p34^{cdc2}$ and cyclin B, whose complex is required for the G_2 to M transition (Draetta, 1990, Draetta *et al.*, 1989, Norbury and Nurse, 1992, Nurse, 1990, Pines and Hunter, 1990). The physiological role of cyclin B has been well established with its degradation being required for the exit from mitosis (Minshull *et al.*, 1989, Murray and Kirschner, 1989, Murray *et al.*, 1989). Other members of the cyclin family have also been shown to associate with $p34^{cdc2}$ or $p34^{cdc2}$-related proteins but the functions of these associations remain largely speculative. Cyclin A associates with cdk2 (Elledge *et al.*, 1992, Rosenblatt *et al.*, 1992, Tsai *et al.*, 1991), a cdc2 homologue, during S phase and G_2 but also associates with $p34^{cdc2}$ (Clarke *et al.*, 1992, Draetta *et al.*, 1989, Pagano *et al.*, 1992, Tsai *et al.*, 1991). Cyclin E has also been shown to be associated with both $p34^{cdc2}$ and cdk2 and was able to activate $p34^{cdc2}$ in G_1 cell extracts (Dulic *et al.*, 1992, Koff *et al.*, 1991, Koff *et al.*, 1992). Recently, several of the D cyclins were shown to complex with multiple kinases closely related to $p34^{cdc2}$ and accumulate in macrophages and in proliferating T-cells (Matsushime *et al.*, 1992, Xiong *et al.*, 1992).

The product of the p13^{suc1} gene in fission yeast is a second protein known to physically associate with p34^{cdc2} either in its monomeric form or when it is associated with cyclins (Booher *et al.*, 1989, Brizuela *et al.*, 1987, Hayles *et al.*, 1986, Hindley *et al.*, 1987, Moreno *et al.*, 1989). Only a small portion of p34^{cdc2} (less than 5%) is bound to p13^{suc1} in the cell (Brizuela *et al.*, 1987, Draetta and Beach, 1988). p13^{suc1} is not a reported substrate of p34^{cdc2} (Brizuela *et al.*, 1987) nor does it appear to activate the kinase activity of pre-activated p34^{cdc2} (Dunphy and Newport, 1989). This association has adopted practical uses for the purification of p34^{cdc2}, by coupling bacterially expressed p13^{suc1} protein to Sepharose beads and eluting with SDS or expressed protein (Dunphy *et al.*, 1988, Labbe *et al.*, 1989, Pondaven *et al.*, 1990). Subsequent modified procedures have also been developed that allow for the isolation of active p34^{cdc2} without elution with p13 protein (Kusubuta, 1992). In addition to yeast p13^{suc1}, homologues have also been identified in higher species (Draetta *et al.*, 1987, Richardson *et al.*, 1990). However, the exact regulatory properties of this subunit with respect to p34^{cdc2} function and activation remain unclear.

In vitro mutagenesis of *S. pombe* p34^{cdc2} specifically targeting charged clusters and known phosphorylation sites for replacement with alanine residues, has revealed distinct regions that are important for binding both p13^{suc1} and cyclins A and B (Brambilla *et al.*, 1991). The N-terminal region of p34^{cdc2} including the glycine motif and lysine 33 involved in ATP binding, as well as a highly acidic stretch of amino acids immediately C-terminal to the glycine motif, were found to be important for cyclin binding. In addition, mutations within the highly conserved PSTAIRE sequence also diminished cyclin binding. Several stretches of basic amino acids scattered throughout the entire cdc2 protein were found to be required for interaction with p13^{suc1} including Lys 20, Arg 22, His 23, Lys 24; Lys 56, Asp 57, Arg 59, His 60; and Arg 215 and Arg 218. It was also apparent from this study

that none of the domains important for cyclin binding overlapped with regions important for $p13^{suc1}$ binding and vice versa.

In addition to the cyclins, the activity of $p34^{cdc2}$ is clearly regulated by phosphorylation events. Initially this stemmed from the fact that fission yeast $p34^{cdc2}$ was shown to be phosphorylated on threonine 167 and tyrosine 15 in a cell cycle dependent manner (Gould *et al.*, 1991, Gould and Nurse, 1989). Abrupt dephosphorylation of tyrosine 15 at mitosis was shown to be required for cdc2 activation, as mutation of this residue to phenylalanine caused cells to prematurely enter mitosis (Gould and Nurse, 1989) and, *in vitro*, tyrosine dephosphorylation of $p34^{cdc2}$ activated its kinase activity (Pondaven *et al.*, 1990). This mechanism appears to be conserved in higher eukaryotes; however, dephosphorylation of an additional residue, threonine 14, acts in conjunction with tyrosine 15 dephosphorylation to activate the kinase (Krek and Nigg, 1991b, Norbury *et al.*, 1991). Both tyrosine 15 and threonine 14 lie within the putative ATP binding motif found in other kinases (Hanks, 1987). It is speculated that phosphorylation of these two residues directly influences the ability of ATP to bind to the kinase (Gould and Nurse, 1989, Nurse, 1990). In the absence of phosphorylation of threonine 14 and tyrosine 15, $p34^{cdc2}$ still requires cyclin B for its activation. Threonine 161 (residue 167 in yeast) is also phosphorylated in a cell cycle dependent manner (Gould *et al.*, 1991, Krek and Nigg, 1991a). Phosphorylation of this residue appears to play a role in allowing $p34^{cdc2}$ to bind to its cyclin B subunits, as mutation of this residue in both yeast and vertebrate $p34^{cdc2}$ prevents cyclin binding (Ducommun *et al.*, 1991, Gould *et al.*, 1991). A fourth residue, serine 277, is also phosphorylated in a cell cycle dependent manner during G_1 and S phases, and is conserved among functional homologues of $p34^{cdc2}$, although the exact effect of its phosphorylation on $p34^{cdc2}$ kinase activity and cyclin binding remains speculative.

Despite the constant level of cdc2 protein during the cell cycle, the amounts of cdc2 mRNA are not constant. Its synthesis and degradation are co-ordinated to maintain protein

levels at a steady state during the cell cycle (Welch and Wang, 1992). With recent evidence for the existence of specific $p34^{cdc2}$ inhibitors (Mendenhall, 1993), the regulation of cdc2 in the cell cycle is clearly becoming much more complicated than investigators had originally envisioned.

$p34^{cdc2}$ Homologues

The identification of $p34^{cdc2}$ as a key cell cycle regulator has led to a surge of research involving the isolation and characterization of cdc2 related proteins in other species, largely through the use of cloning techniques. This work has resulted in the division of the cdc2 family into at least two different subgroups. The first subgroup consists of functional homologues, those able to complement cdc2/CDC28 temperature sensitive mutations in yeast, and includes cdc2 genes cloned from human (Lee and Nurse, 1987), chicken (Krek and Nigg, 1989), Xenopus (Milarski *et al.*, 1991), mouse (Cisek and Corden, 1989), Drosophila (Jimenez *et al.*, 1990, Lehner and O'Farrell, 1990), and a variety of plant species (Colasanti *et al.*, 1991, Hirayama *et al.*, 1991, Hirt *et al.*, 1991). Two other functional homologues, able to complement cdc28 temperature sensitive mutations, have also been cloned (Elledge and Spottswood, 1991, Ninomiya *et al.*, 1991). Cdk2 (cyclin dependent kinase) and cdk3, so named because of their ability to associate with cyclin proteins, are also thought to play a role in cell cycle control along with $p34^{cdc2}$ (Elledge *et al.*, 1992, Meyerson *et al.*, 1992, Tsai *et al.*, 1991). In addition to both functional complementarity and cyclin binding, both cdk2 and cdk3 are able to bind to $p13^{suc1}$ (Meyerson *et al.*, 1992). In the case of cdk2, it is likely that this kinase plays a role in controlling G_1/S phase of the cell cycle, as depletion of cdk2 blocks DNA synthesis (Fang and Newport, 1991) and activation of its kinase activity occurs prior to the onset of S-phase (Pagano *et al.*, 1992, Rosenblatt *et al.*, 1992). The conclusive assignment of cdk3 in cell cycle related functions awaits further studies.

Sequence alignment of these functional homologues has revealed highly conserved motifs in addition to those characteristic of serine/threonine kinases (Hanks *et al.*, 1988). In the N-terminus of the protein, a sequence region surrounding the putative ATP binding domain is completely conserved in functional cdc2 homologues, including what corresponds to the two regulatory phosphorylatable residues in mammalian $p34^{cdc2}$, threonine 14 and tyrosine 15. Two other potentially regulatory phosphorylation sites, threonine 161 and serine 277, are also completely conserved. A second highly conserved sequence has been termed the PSTAIRE region. Additional sequences include LWYR motif and GDSEIDQ corresponding to amino acids 165-168 and 206-211 of the human cdc2, respectively. Sequences in the extreme C-terminal regions of these proteins are more divergent.

Members of the second subgroup of the cdc2 family although still similar in sequence to $p34^{cdc2}$, are either unable to complement temperature sensitive mutations in yeast, or their ability to complement has not been tested. Certain members of this subgroup appear to have a function that is unrelated to the cell cycle. Many of these homologues possess N-terminal and/or C-terminal extensions in addition to what typically comprises the $p34^{cdc2}$ catalytic domain. In budding yeast, *S. cerevisiae,* several cdc2 related kinases have been discovered whose function appears to be involved in cell growth and division (Courchesne *et al.*, 1989, Elion *et al.*, 1990, Irie *et al.*, 1991, Toh *et al.*, 1988). Several protein kinases found in human and mouse cDNA libraries, whose notable differences from $p34^{cdc2}$ include amino acid changes within the PSTAIRE region, have recently been cloned. The majority of these homologues show restricted and varied patterns of tissue distribution (Meyerson *et al.*, 1992, Okuda *et al.*, 1992), but their potential function remains elusive. Additional homologues include p58, isolated from a human liver cDNA library whose overexpression results in prolonged G_1 phase and telophase (Bunnell *et al.*, 1990), and CLK, a protein kinase having homology to $p34^{cdc2}$, but possessing intrinsic

serine/threonine and tyrosine kinase activity (Johnson and Smith, 1991). MAK is a germ cell associated kinase expressed in a tissue and stage specific manner displaying significant homology to $p34^{cdc2}$ and is speculated to play a role in spermatogenesis (Matsushime *et al.*, 1990). PSK-J3, originally isolated by Steven Hanks by screening a cDNA library with probes selected from homologous regions in protein kinases (Hanks, 1987), was more recently shown to associate with cyclin D (Matsushime *et al.*, 1992). Evidence suggesting the involvement of cdc2 related kinases in processes unrelated to the cell cycle is only beginning to emerge. CHED, a cdc2 homologue that appears to be involved in hematopoiesis (Lapidot *et al.*, 1992), has been isolated. More recently, a cdc2-like kinase has been cloned and found to be highly expressed in terminally differentiated neurons (Hellmich *et al.*, 1992). There is also evidence to suggest that cdc2 or related proteins may be involved in platelet function (Lerea, 1992, Samiei *et al.*, 1991). The MAP or ERK kinase family is also related to $p34^{cdc2}$, with $p44^{erk1}$ sharing approximately 33% identity with human $p34^{cdc2}$ (Pelech and Sanghera, 1992). Members of this family of kinases are not only activated at M-phase during mitosis, but are also activated in response to a wide variety of stimuli including growth factors, mitogens and differentiating agents (Pelech and Sanghera, 1992).

$p34^{cdc2}$ Substrates

Given the vast number of phosphorylation events that take place during the cell cycle, the ability to distinguish proteins phosphorylated by $p34^{cdc2}$ or other closely related kinases becomes increasingly difficult. Many proteins have been identified as *in vitro* targets of $p34^{cdc2}$ with a subset having identical phosphorylation sites *in vivo*. These substrates can be grouped into several classes as shown in Table 1, based on what is presently known about their respective functional properties.

The first class of $p34^{cdc2}$ substrates can be classified as structural proteins. Histone H1 has traditionally been used as a means of assaying $p34^{cdc2}$ kinase activity. Histone H1 is phosphorylated on multiple sites (Langan, 1982), and its *in vivo* phosphorylation sites by the growth associated histone H1 kinase (a complex of $p34^{cdc2}$ and cyclin B) have been identified (Langan *et al.*, 1980). These are found in the basic regions at the N- and C-termini of histone H1 and are also regions that appear to be involved in chromosome condensation (Reeves, 1992). Although the functional significance of histone H1 phosphorylation has not yet been firmly established, it is thought that phosphorylation of these sites may alter the packing of the nucleosome and therefore contribute to chromosome condensation (Zlantanova and Yaneva, 1992).

Lamins are intermediate filament proteins whose phosphorylation levels increase during mitosis, and make up the nuclear lamina, a mesh of fibrillary proteins that underlie the nuclear membrane. Peter and co-workers have shown that $p34^{cdc2}$ phosphorylates B-lamins on sites identical to those phosphorylated *in vivo* and is able to induce lamin disassembly upon incubation with isolated nuclei suggesting that mitotic disassembly of the nuclear lamina results from direct phosphorylation of lamins by $p34^{cdc2}$ (Peter *et al.*, 1990b). Heald and McKeon showed that mutations affecting these sites interfered with lamin disassembly (Heald and McKeon, 1990). In addition, a chicken lamin protein when expressed in yeast was shown to be phosphorylated by a mitotically active kinase that was temperature sensitive in cdc2 temperature sensitive mutant strains (Enoch *et al.*, 1991). More recently, the same group has shown that lamin B2 head to tail polymers reconstituted *in vitro* from bacterially expressed protein when phosphorylated by $p34^{cdc2}$, disassembled into dimers and that this effect was reversed by treatment with phosphatase (Peter *et al.*, 1991).

Two major nucleolar proteins, NO38 and nucleolin, are highly phosphorylated *in vivo* during mitosis and are phosphorylated on identical sites *in vitro* by $p34^{cdc2}$. Nucleolin

is considered to be a multi-functional protein acting during the synthesis and processing of ribosomal RNA, while NO38 is a nuclear matrix protein (Peter *et al.*, 1990a). Although the significance of their phosphorylation is unknown, it is speculated that their post-translational modification might play some function in the disassembly of the nucleolus during mitosis.

Vimentin is another type of intermediate filament protein which is hyperphosphorylated and depolymerized as cells enter mitosis. Chou and coworkers have purified an enzyme from rat BHK cells consisting of $p34^{cdc2}$ and 65 and 110kDa subunits. They showed that this purified kinase phosphorylates vimentin *in vitro* on a subset of sites phosphorylated *in vivo* during mitosis and the *in vitro* phosphorylation of vimentin caused their disassembly (Chou *et al.*, 1990, Chou *et al.*, 1991). Interestingly, the neurofilaments (NF), another intermediate filament protein expressed specifically in neuronal cells, is phosphorylated *in vitro* by starfish $p34^{cdc2}$, a complex of cyclin B and the starfish homologue of $p34^{cdc2}$, at the C-terminal domain resulting in the dissociation of NF-H from microtubules (Hisanaga *et al.*, 1991). In addition, Guan *et al.* have shown that a complex of cyclin A and $p34^{cdc2}$ purified from rat pheochromocytoma also phosphorylates NF-H, NF-L and NF-M but does not cause filament disassembly (Guan *et al.*, 1992).

Caldesmon, a regulatory protein implicated in the control of contractility and motility in non-muscle and smooth muscle cells (Bretscher, 1986) is phosphorylated *in vitro* by $p34^{cdc2}$ (Mak *et al.*, 1991b, Yamashiro *et al.*, 1991). In the case of non-muscle caldesmon, $p34^{cdc2}$ *in vitro* phosphorylation sites were identical to those phosphorylated *in vivo* during mitosis and phosphorylation was found to reduce binding affinity for both actin and calmodulin and caused caldesmon to dissociate from microfilaments in cultured fibroblasts (Yamashiro *et al.*, 1991). *In vitro* phosphorylation sites in smooth muscle caldesmon have also been mapped with the majority of sites residing in the C-terminal domain of caldesmon known to bind actin and calmodulin (Mak *et al.*, 1991a), suggesting

that phosphorylation by $p34^{cdc2}$ may contribute to major structural reorganizations that take place during mitosis. It has also been reported that $p34^{cdc2}$ is able to phosphorylate myosin II regulatory chains on three sites previously shown to inhibit the actin activated myosin ATP'ase of smooth muscle and non-muscle myosin (Lewin, 1990, Pines and Hunter, 1990, Satterwhite *et al.*, 1992).

Two tumor suppressor proteins, both phosphorylated in a cell cycle dependent manner, have also been identified as *in vitro* substrates of $p34^{cdc2}$. $p110^{Rb}$ is a nuclear phosphoprotein that is phosphorylated from S to M phase and dephosphorylated in G_1. Lin and co-workers showed that human $p34^{cdc2}$ immunoprecipitated from mitotic cells and purified MPF were able to phosphorylate $p110^{Rb}$ on sites corresponding to *in vivo* phosphorylation sites (Lin *et al.*, 1991). A second tumor suppressor protein, p53, is also phosphorylated by $p34^{cdc2}$ on a single serine residue that is also phosphorylated *in vivo* (Bischoff *et al.*, 1990). Although the consequences of these phosphorylations have yet to be verified it is thought that their post-translational modification by $p34^{cdc2}$ might regulate their anti-proliferative activity.

Several tyrosine and serine/threonine kinases are *in vitro* substrates of $p34^{cdc2}$. David Litchfield and coworkers have reported the *in vitro* phosphorylation of both α and β subunits of casein kinase II but were unable to detect any alteration in casein kinase II activity (Litchfield *et al.*, 1991, Litchfield *et al.*, 1992). In contrast, another group has demonstrated that *in vitro* phosphorylation of the β subunit of casein kinase II by $p34^{cdc2}$ stimulates its activity (Mulner *et al.*, 1990). Two non-receptor tyrosine kinases, $pp60^{c\text{-}src}$ and $p150^{c\text{-}abl}$, are also *in vitro* substrates of $p34^{cdc2}$. $pp60^{c\text{-}src}$ is phosphorylated *in vivo* on two threonine residues and one serine residue in the N-terminal region of $pp60^{c\text{-}src}$ at mitosis and *in vivo* these modifications are associated with a four to seven fold increase in $pp60^{c\text{-}src}$ kinase activity (Chackalaparamil and Shalloway, 1988). Purified $p34^{cdc2}$ was shown to phosphorylate these sites *in vitro* although an increase in $pp60^{c\text{-}src}$ tyrosine kinase

Table 1: Identified *In Vitro* Substrates of $p34^{cdc2}$

Substrate	Same sites Phosphorylated *in vivo*	Sequence Information
Structural Proteins		
Histone H1	yes	S/T-P-X-K
Lamin B2	yes	S-P-T-R
NO38, Nucleolin	yes	T-P-X-K-K
Vimentin	yes	S-S-P-G-G
		S-A-L-R-P
Neurofilament	?	?
Caldesmon		
non-muscle	yes	S-P-G-G-T
		G-T-P-N-K-K
smooth muscle	?	S-S-K-R
DNA Binding Proteins		
SV40 Lg T	yes	S-T-P-P-K-K-R
RPA	yes	Q-S-P-G-G-F-G
		G-S-P-A-P-S-Q
RNA Pol II	?	Y-S-T-P-S-P-S
HMG I	yes	P-T-P-K-R-P-G
Tumor Suppressor Proteins		
p53	yes	S-P-Q-P-K-K-K
$p110^{Rb}$	yes	?

Table 1 (continued) Identified *In vitro* Substrates of $p34^{cdc2}$

Substrate	Same Site(s) Phosphorylated *in vivo*	Sequence Information
Protein Kinases		
$pp110^{c\text{-}abl}$	yes	S-P-L-L-P-R
$pp60^{c\text{-}src}$	yes	S-Q-T-P-N-K-T
		H-R-T-P-S-R-S
		V-T-S-P-Q-R-A
Casein Kinase II		
α-subunit	yes	?
β-subunit	yes	F-K-S-P-V-K-T
Other Proteins		
Rab	?	Q-S-T-P-V-K
		R-S-P-R-R-T-Q
EF-1β	?	?
Cyclin B	?	S-P-E-P
		S-P-S-P
		R-S-P-L-P
		S-P-V-M

activity was not demonstrated (Shenoy *et al.*, 1989). $pp60^{c\text{-}src}$ is associated with cytoskeletal structures near the plasma membrane and its phosphorylation by $p34^{cdc2}$ might imply some role for $pp60^{c\text{-}src}$ as a mediator of cytoskeletal rearrangement. $p150^{c\text{-}abl}$ is phosphorylated on three sites during interphase and seven additional sites during mitosis (Kipreos and Wang, 1990). Kipreos and Wang have shown that two interphase sites and all of the mitotic sites are phosphorylated *in vitro* by $p34^{cdc2}$ isolated from mitotic or interphase cells. As was the case with $pp60^{c\text{-}src}$, these phosphorylations did not contribute to an increase in $p150^{c\text{-}abl}$ tyrosine kinase activity *in vitro*, although physiologically this represents a potential means for regulating its activity.

Several DNA binding proteins have also been identified as *in vitro* substrates of cdc2 kinase implicating the involvement of one or more forms of $p34^{cdc2}$ in the control of S phase. McVey and co-workers have shown that $p34^{cdc2}$ purified from HeLa cells was able to phosphorylate SV40 large T antigen on threonine 124, a residue also phosphorylated *in vivo* , and was able to bind to the viral origin of replication and initiate DNA replication *in vitro* (McVey *et al.*, 1989). Dutta and Stillman have purified a kinase consisting of a complex of cyclin B and $p34^{cdc2}$ that phosphorylates RPA, a single stranded DNA binding protein complex that is essential for initiation and elongation stages of SV40 DNA replication (Dutta and Stillman, 1992). Cisek and Corden have also purified a kinase, the mouse homologue of $p34^{cdc2}$, that phosphorylated the highly repetitive carboxy-terminal domain of RNA polymerase II, implicating a role for cdc2 in transcriptional regulation (Cisek and Corden, 1989). Several members of the high mobility group proteins, believed to be important structural components affecting chromatin structure by binding to A-T rich sequences in DNA, were also identified as *in vitro* substrates of $p34^{cdc2}$ (Meijer *et al.*, 1991, Nissen *et al.*, 1991, Reeves *et al.*, 1991). Their phosphorylation by $p34^{cdc2}$ resulted in a decrease in binding affinity of these proteins for A-T rich DNA sequences.

Additional substrates have been identified, including several GTP binding proteins belonging to the Rab family (Bailly *et al.*, 1991) and elongation factor γ, involved in protein biosynthesis (Belle *et al.*, 1989, Janssen *et al.*, 1991, Mulner *et al.*, 1989). Cyclin B, which constitutes the major regulatory component for mitotic cdc2, has also been shown to be an *in vitro* substrate, being autophosphorylated by purified MPF (Gautier *et al.*, 1990). For sea urchin cyclin B, sites phosphorylated have been tentatively identified by site directed mutagenesis in the N-terminal region of cyclin B and are shown in Table 1 (Pines and Hunter, 1990). In addition, Izumi and Maller replaced potential phosphorylation sites in Xenopus cyclins B1 and B2 and found that Ser 90 of cyclin B2 and Ser 94 and 96 of cyclin B1 were the main phosphorylation sites in functional Xenopus extracts and when phosphorylated *in vitro* with purified MPF (Izumi and Maller, 1991).

Consensus Motifs

Our understanding of the importance of protein phosphorylation in the control and regulation of cellular processes has made significant progress. Central to this theme is determining how enzymes selectively recognize their respective substrate proteins. Many contributions in the protein kinase field have indicated that protein kinases recognize and phosphorylate discrete sites on their target proteins and that their ability to do this is primarily dependent on the local primary structure surrounding the phosphorylatable residue (Kemp and Pearson, 1990).

With a knowledge of primary sequences surrounding the phosphorylatable residue on a substrate, the elucidation of specificity determinants involves modelling synthetic peptides after the phosphorylation site sequences and characterizing the kinetics of phosphorylation of peptides. Peptide analogues that possess similar kinetic properties to those of the native protein are considered to contain all of the necessary determinants for efficient recognition and phosphorylation by the enzyme. These then can be used as a basis

for the investigation of specific determinants by systematically substituting residues within the peptide sequence. Sequence elements immediately surrounding the phosphorylation site that are considered essential for recognition and phosphorylation by the kinase are collectively referred to as a consensus sequence (Kennelly and Krebs, 1991). Using synthetic peptides, consensus sequences for numerous protein kinases have been determined and representative examples are shown in Table 2 (Kennelly and Krebs, 1991). The cAMP-dependent protein kinase for example, phosphorylates serine and threonine residues in synthetic peptides that have basic amino acid residues at the -2 and -3 positions relative to the phosphorylatable residue (position 0) (Kemp, 1990). Casein kinase II, on the other hand, requires the presence of acidic residues C-terminal to the phosphorylatable residue with the optimal position being +3 (Kemp, 1990). Although consensus sequences provide a powerful tool in terms of being able to predict potential substrates and their phosphorylation sites, it should be noted that the presence of a consensus sequence in a protein does not guarantee that the protein is phosphorylated by that particular kinase, nor does it indicate conclusively the kinase responsible for the phosphorylation *in vivo*. Indeed higher order structures that small peptides are unable to adopt must also play some role in enzyme substrate interaction. The consensus sequence can only serve as a guideline for the identification of potentially physiological substrates. For $p34^{cdc2}$, a consensus sequence has been proposed by numerous groups, based on the sequence analysis of a variety of proteins known to be phosphorylated *in vitro* by $p34^{cdc2}$ and whose same site(s) are phosphorylated *in vivo* (Draetta, 1990, Langan *et al.*, 1989, Langan *et al.*, 1980, Maller, 1990, Moreno and Nurse, 1990, Peter *et al.*, 1990a, Pines and Hunter, 1990). This sequence information, which is summarized in Table 2, reveals what is generally agreed to be the consensus sequence for $p34^{cdc2}$ consisting of **S/T**-P-X-K/R where X is a polar amino acid, but has yet to be verified with synthetic peptides. It has also been suggested that the only recognizable motif is a phosphorylatable residue followed by a proline

Table 2: Consensus Sequence Motifs for Selected Protein Kinases

Serine/Threonine Kinase	Consensus Motif
cAMP Dependent protein kinase	X-X-R-X-S-X
PKC(α, β, γ)	X-R-X-X-S-X-R-X
Phosphorylase kinase	K-R-K-Q-I-S-V-R
MLCK	X-K-K-R-X-X-R-X-X-S-X-X
Casein kinase I	X-E-X-X-S-X
Casein kinase II	X-S-X-X-E-X
MAP Kinases	P-X-S/T-P

(Draetta, 1990). Proline is known to exert structural effects similar to a bend or a kink in protein molecules and the predicted secondary structure of the consensus sequence is a β turn (Moreno and Nurse, 1990, Suzuki, 1989). In addition this motif has been shown to be a DNA binding motif (Suzuki, 1989). As shown in Table 1, there are clearly exceptions to this motif. Many of the identified sequences of substrate proteins lack a basic residue, and clearly it appears that amino acids other than polar residues can be tolerated in the +2 position. In addition, the *in vitro* phosphorylation sites by $p34^{cdc2}$ identified in myosin light chain do not have the C-terminal proline, but do have basic residues. Pines and Hunter have suggested that these sites are phosphorylated because the serine residues that are phosphorylated in myosin light chain are predicted to lie on a β-turn in the protein and thus may adopt a similar conformation to that in which the serine would be followed immediately by a proline (Pines and Hunter, 1990). A minor $p34^{cdc2}$ phosphorylation site has also been identified in vimentin which does not contain a C-terminal proline adjacent to the phosphorylatable residue, although the presence of a contaminating kinase activity that phosphorylated this site was not ruled out by the authors (Chou *et al.*, 1991).

A family of 42 to 44kDa serine/threonine kinases distantly related to the cdc2 kinase family in sequence but whose regulatory properties have significantly diverged are the MAP kinases or mitogen activated protein kinases (Pelech and Sanghera, 1992 for review). The *in vitro* substrate specificity determinants for various members of this family have been elucidated using synthetic peptides derived from myelin basic protein or threonine 669 containing peptides derived from the EGF receptor (Clark-Lewis *et al.*, 1991, Gonzalez *et al.*, 1991). A consensus motif similar to that of $p34^{cdc2}$, consisting of P-X-S/T-P, was defined. In these studies, X was ideally a basic or neutral amino acid for $p42^{mpk}$ and $p44^{erk1}$, whereas $p44^{mpk}$ could also tolerate an acidic residue. The proline immediately C-terminal to the phosphorylatable residue was found to be essential for MAP kinase activity whereas the proline located at the -2 position, although not essential, was favored for

efficient substrate phosphorylation by MAP kinases (Clark-Lewis *et al.*, 1991, Gonzalez *et al.*, 1991). Given the overlap of the MAP kinase consensus sequence with the proposed consensus sequence for $p34^{cdc2}$, it is not surprising that some $p34^{cdc2}$ substrates are also targets of the MAP kinases and vice versa. Indeed, lamin B2, phosphorylated on serine 16 by $p34^{cdc2}$, is also phosphorylated on the same site by MAP kinase and both kinases are able to induce lamin disassembly *in vitro* (Peter *et al.*, 1992).

Different Forms of Cdc2 Have Different Substrate Preferences

Cyclins have been proposed to act on their cdc2 catalytic subunits to regulate their substrate specificity by targeting their catalytic subunits to a particular substrate (Pines and Hunter, 1990). There is also evidence to suggest that cyclins may influence the subcellular distribution of their associated kinase subunits and thus confer a certain degree of specificity by targeting the catalytic subunit to a particular region of the cell (Pines and Hunter, 1991b). Evidence implicating the influence of cyclins on the substrate specificity of their cdc2 catalytic components has been well documented. Brizuela and coworkers were able to fractionate high molecular weight complexes of human histone H1 kinase, known to contain cyclin B and $p34^{cdc2}$, from corresponding low molecular weight complexes lacking cyclin B. They found that both the high and low molecular weight forms were able to phosphorylate casein, but only the high molecular weight form phosphorylated histone H1 (Brizuela *et al.*, 1989). In another study using antibodies specific for Xenopus cyclin A, B1 and B2, Minshull and coworkers demonstrated that histone 2B was a much better substrate for the cyclin A-cdc2 complexes over cyclin B-cdc2 complexes, although the sequences surrounding the phosphorylatable residues of both histones were identical (Minshull *et al.*, 1990). Using antibodies for cdk2 or the human homologue of $p34^{cdc2}$ (cdc2Hs), Elledge and his coworkers reported that both formed complexes with cyclin A and phosphorylated histone H1 equally well, but that cdc2Hs-cyclin A complexes

preferentially phosphorylated another *in vitro* substrate, RF-A, 10 fold better than cdk2-cyclin A complexes (Elledge *et al.*, 1992). Matshushime has recently identified complexes of cyclin D and cdk4 using anti-cyclin antibodies that do not possess any histone H1 kinase activity, but that are able to phosphorylate the retinoblastoma gene product (Matsushime *et al.*, 1992). Hall and his co-workers have purified a growth factor sensitive proline-directed kinase from rat pheochromocytoma cells and have shown that it contains a complex of $p34^{cdc2}$ and cyclin A (Hall *et al.*, 1991) and unlike the histone H1 kinase it is able to phosphorylate glycogen synthase (Erikson and Maller, 1989). More recently, $p13^{suc1}$ was shown to suppress the phosphorylation of two intermediate filament proteins, vimentin and desmin, but did not affect histone H1 phosphorylation (Kusubuta, 1992). The continual expansion of the cdc2 kinase family and the discovery of a variety of cyclins provides numerous opportunities for diversity of function and regulation of these enzymes both within the cell cycle and potentially in other cellular processes. While it is possible that various cdc2 related kinases with their cyclin components have a unique substrate specificity, detailed enzymatic and biochemical characterizations of the majority of enzyme species in this growing family are not yet available.

Recently, in our laboratory, an enzyme has been purified from bovine brain based on its ability to phosphorylate a synthetic peptide derived from the three *in vitro* $p34^{cdc2}$ phosphorylation sites of $pp60^{c\text{-}src}$ (R-R-P-D-A-H-R-T-P-N-R-A-F). The purified form of this kinase consists of a 33kDa subunit and a 25kDa subunit. The activity of this kinase is clearly proline dependent, as phosphorylation of the pro-src peptide containing alanine replaced for the proline at the +1 position is not phosphorylated (Lew *et al.*, 1992a). In addition to the absolute requirement for a C-terminal proline, both the brain proline directed kinase and $p34^{cdc2}$ were shown to phosphorylate neurofilaments H and M and smooth muscle caldesmon on identical sites, as deduced from peptide mapping experiments, suggesting that the two enzymes possess a similar substrate specificity (Lew *et al.*, 1992b)

(Alan Mak, personal communication). The 33 kDa subunit has recently been cloned and besides containing all identifiable hallmarks associated with the protein kinases (Hanks *et al.*, 1988), it was found to be similar in sequence to both $p34^{cdc2}$ and cdk2.

The purification of this enzyme from bovine brain has provided large quantities for enzymatic and biochemical characterization. The primary focus of this study involves determining the substrate specificity requirements of the brain PDK by amino acid substitution of synthetic peptides derived from a $p34^{cdc2}$ phosphorylation site in histone H1 (Felix *et al.*, 1990) and characterizing their respective kinetic parameters.

EXPERIMENTAL PROCEDURES

Materials - [γ-^{32}P]-ATP and p-nitro blue tetrazolium chloride (NBT) were purchased from ICN Biomedicals. Phosphocellulose P81 paper was purchased from Whatman. Polyvinylidene fluoride membranes and Immobilon-P were purchased from Millipore. Hydroxylapatite, Affigel-10 and molecular weight markers were obtained from Bio-Rad. DEAE Sepharose CL-6B, CNBr activated Sepharose, and Sephadex G10 were purchased from Pharmacia. NP-40 was purchased from Fluka Biochemicals. Leupeptin and pepstatin A were purchased from United States Biochemical Corporation. Goat anti-(rabbit IgG-alkaline phosphatase conjugated was purchased from Jackson Immuno Research. Isopropyl-1-thio-β-D-galactopyranoside, DMEM and fetal bovine serum were purchased from Gibco BRL. m-Maleimido-benzoyl-N-hydroxysuccinimide ester and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were purchased from Pierce. KLH was obtained from Calbiochem. HEPES, SDS and Tris were obtained from Boehringer Mannheim. Fmoc amino acids were purchased from Bachem. p-Hydroxymethyl-phenoxymethyl polystyrene resin was purchased from Applied Biosystems Inc. Solvents for peptide synthesis were obtained from the following suppliers: trifluoroacetic acid, piperidine, dimethylaminopyridine, N,N-dimethylformamide, N,N-dicyclohexyl-carbodiimide, 1-hydroxy-benzotriazol and (2-(1H-benzotriazol-1-yl)-1,1,3,3,tetra-methyl-uroniumhexafluorophosphate) were purchased from Applied Biosystems Inc. Dichloromethane and N-methylpyrridone were purchased from Burdick and Jackson. Methanol was purchased from BDH Inc. Myosin light chain kinase pseudosubstrate peptide was purchased from Peninsula Laboratories. Histone H1, purified from winter flounder, was a generous gift from Dr. A. Mak (Queen's University, Canada). Smooth muscle myosin light chain kinase, protein kinase C and the PKC pseudosubstrate peptides

were kindly provided by Dr. M. P. Walsh (University of Calgary). All other materials were purchased from Sigma.

Peptides - The pro-src peptide was synthesized as described previously (Lew *et al.*, 1992a). Histone derived peptides were synthesized using an Applied Biosystems 431A peptide synthesizer. Peptides were synthesized from protected Fmoc amino acids (Carpino and Han, 1970, Carpino and Han, 1972), and deprotected and cleaved from the resin using 95% (v/v) TFA in water for 1.5 hours at room temperature. After separation of cleaved peptides from the resin by filtration, and washing of the resin with TFA and dichloromethane, the filtrate and washes were concentrated to a 1ml volume on a rotary evaporator, and the peptide was precipitated out of solution by the addition of 50ml cold diethyl ether. The precipitate was collected on polyvinylidene membranes and resuspended in a minimal volume of 20% (v/v) acetic acid in water. Peptides were subsequently purified on a Sephadex G-10 column and eluted with 20% (v/v) acetic acid in water. The purity of all peptides was analyzed by HPLC on a Vydac 218TP54 C_{18} reversed phase analytical column in a solvent system consisting of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B). Samples were eluted from the column at a flow rate of 1ml/min on a linear gradient from 100% solvent A, 0% solvent B to 60% solvent A, 40% solvent B in 40 minutes. Peptides were found to be greater than 95% pure. Quantitative amino acid analysis of purified peptides was determined using a Beckman model 6300 amino acid analyzer using norleucine as an internal standard.

Preparation of C-terminal, PSTAIRE and N-terminal Antibodies -Purified synthetic peptides derived from human $p34^{cdc2}$, N-terminal (cys-cdc2(8-20)), PSTAIRE (cys-cdc2(42-57)) or C-terminal (cys-cdc2(288-297)), were coupled to keyhole limpet hemocyanin through the additional cysteine residues of the peptides with m-maleimido-benzoyl-N-hydroxysuccinimide ester as a coupling reagent according to the standard methods (Harlow and Lane, 1988). New Zealand rabbits were immunized according to the

standard methods and serum was collected. After a 50% ammonium sulphate precipitation, antibodies were purified on an Affigel 10 column coupled with the appropriate peptide according to standard protocols (Harlow and Lane, 1988). Purified antibodies were tested with affinity purified $p34^{cdc2}$ and stored at -70°C

Preparation of Bacterially Expressed $p13^{suc1}$ - The $p13^{suc1}$ protein, used for affinity purification of $p34^{cdc2}$, was prepared from an overexpressing strain of *Escherichia coli*, a gift from R.Golsteyn and Dr. T. Hunt at Cambridge University, essentially as described by Brizuela *et al.* (Brizuela *et al.*, 1987). Over expressing *E. coli* was grown in LB medium containing 50µg/ml ampicillin. On reaching A_{600} of 0.5, cultures were induced with IPTG for 3.5 hours. Cells from a 1 liter culture were harvested by centrifugation at 14,000xg and lysed in 50ml of homogenization buffer containing 20mM HEPES, pH 7.0, 2mM EDTA, 1mM DTT, 1mM PMSF and 1mg/ml lysozyme for one hour and sonicated briefly at 150 watts. Cell lysates were centrifuged at 100,000xg for one hour and the supernatant dialysed against homogenization buffer. The p13 protein was loaded onto a DEAE CL-6B column equilibrated in homogenization buffer and eluted with a linear gradient of NaCl (0-200mM). p13 was further purified on an S200 gel filtration column in homogenization buffer. Samples containing purified p13 were pooled and dialyzed against deionized water, pH 8 and the lyophilized protein was stored at -20°C. p13 was coupled to CNBr-activated Sepharose according to the manufacturer's instructions.

Preparation of HeLa Cell $p34^{cdc2}$ and the Brain PDK- HeLa cell $p34^{cdc2}$ isolated from colcemid treated cells was partially purified essentially as described by Pondaven *et al.* (Pondaven *et al.*, 1988). HeLa cells were grown in DMEM media supplemented with 10% fetal bovine serum at a density of $2\text{-}4x10^8$ cells/ml and were treated with colcemid (100µg/L) for 18 hours prior to being harvested. The pelleted cells were stored at -70°C prior to use. Cells were lysed in lysis buffer containing 12.5mM MOPS, pH 7.2, 15mM $MgCl_2$, 15mM EGTA, 0.1%(w/v) NP-40 and protease inhibitors leupeptin (10µg/ml),

pepstatin A (10μg/ml) soybean trypsin inhibitor (10μg/ml), benzamidine (100μM) and PMSF (1mM) at 4°C. Cells were then sonicated for 4 five second bursts at 75W and incubated on ice for 15 minutes. Cell lysates were clarified by centrifugation at 100,000xg for 45 minutes. The supernatant was loaded onto a 5 ml p13 column batchwise that had been washed with bead buffer: 50mM Tris pH 7.4, 250mM NaCl, 1mM EGTA, 1mM EDTA and 0.1%(v/v) NP-40 and protease inhibitors as described above. After incubating for 45 minutes, at 4°C, the column was drained, washed with bead buffer and eluted with 5ml of 1.5mg/ml bacterially expressed p13 in bead buffer and protease inhibitors. The p13 column was washed and the HeLa cell 100,000xg supernatant reloaded onto the p13 column and the procedure repeated a second time. The eluted material from the p13 column was subsequently loaded onto a 1ml hydroxylapatite column that had been equilibrated with buffer D consisting of 12.5mM MOPS, pH 7.2, 7.5mM $MgCl_2$ and 1mM EGTA to remove excess p13. The column was washed with buffer D and eluted with 0.5M phosphate in buffer D. Fractions were assayed for histone peptide kinase activity, pooled and dialyzed against 25mM Tris pH 7.6 and 1mM EGTA and stored at 4°C.

The bovine brain proline directed kinase (PDK) was purified according to the procedure described by Lew *et al.* (Lew *et al.*, 1992a). For kinetic assays, PDK was diluted appropriately in 20mM MOPS pH 7.4, containing 1mg/ml bovine serum albumin.

Gel Electrophoresis and Western Blotting - SDS-PAGE was performed according to the procedure described by Laemmli (Laemmli, 1970) and Western blotting was performed according to the method of Towbin *et al.* (Towbin *et al.*, 1979) using alkaline phosphatase-linked secondary antibody and p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the color substrates.

Phosphorylation Reactions - Reaction mixtures were essentially as described previously (Lew *et al.*, 1992a) and contained in a final volume of 30 μl: 20mM MOPS pH 7.4, 5mM $MgCl_2$, 100μM [γ^{32}P]-ATP (500-1500 dpm/pmol), peptide substrate as indicated and

protein kinase. Reactions were started with the addition of enzyme and carried out for 10 or 30 minutes at 30°C depending on the peptide substrate. Assays were terminated with the addition of 15µl of 50% (v/v) acetic acid in water. Duplicate 20µl samples were spotted onto phosphocellulose paper and the papers were washed with 6x500 ml of 0.3% H_3PO_4, rinsed in acetone and dried. Phosphate incorporation into labelled peptides was quantitated by liquid scintillation using a Beckman LKB 1215 scintillation counter.

Analysis of Stoichiometry - Phosphorylation reactions were carried out as described above in 60µl assay volume containing 20µM peptide. A 30µl sample was taken at 30 min, 15µl of 50% acetic acid was added and duplicate 20µl samples were spotted onto P81 paper. 3.8µl of fresh enzyme and [γ^{32}P]-ATP were added, and phosphorylation reactions were continued for another 30 minutes, at which time a second 30µl aliquot was taken and spotted onto P81 paper.

Determination of Kinetic Constants - Kinetic constants were determined from Lineweaver-Burke plots using linear regression analysis. Each K_m and V_{max} value was determined at least twice with a minimum of 8 different peptide concentrations per determination. K_m values are reported as the mean values. Relative V_{max} values, in pmol/min/µl of purified enzyme, were expressed as a percentage of control peptide and were normalized for each batch of enzyme used, as protein concentrations of the purified enzyme were too low to measure by the standard methods.

RESULTS

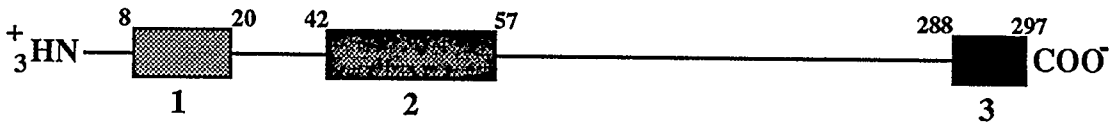
Immunological Characterization of Bovine Cytosolic Extracts and Purified Brain PDK

The existence of significant levels of $p34^{cdc2}$ in brain has been previously reported (Draetta *et al.*, 1988). Speculation that $p34^{cdc2}$ in a such highly non-proliferative tissue may have functions potentially unrelated to the cell cycle prompted us to investigate the existence of $p34^{cdc2}$ or immunologically related proteins in the cytosolic extracts from bovine brain. We have three polyclonal antibodies that are directed against peptide sequences from distinct regions of HeLa cell $p34^{cdc2}$. A schematic representation of the epitopic sites for these three antibodies is shown in Figure 1. The N-terminal antibody (N1-Ab) is derived from a peptide sequence in $p34^{cdc2}$ that contains the G-X-G-X-X-G motif which constitutes a portion of the ATP binding site and also contains two highly conserved regulatory phosphorylation sites, threonine 14 and tyrosine 15 (Lew *et al.*, 1992a). The PSTAIRE antibody is directed towards a 16-mer peptide sequence corresponding to this highly conserved sequence region in functional cdc2 homologues (Lee and Nurse, 1987). The C-terminal antibody (C1-Ab) is directed against the last 10 amino acid residues of human $p34^{cdc2}$, a sequence region that is unique in sequence in comparison to other $p34^{cdc2}$ homologues (Draetta and Beach, 1988). HeLa cell $p34^{cdc2}$, partially purified from whole cell lysates according to the protocol described in the "Experimental Procedures", was used as a positive control for all Western blots.

Cytosolic extracts, containing equal amounts of protein from bovine brain, were resolved by SDS-PAGE and analyzed by Western blot using C-terminal, N-terminal or PSTAIRE antibodies. As shown in Figure 2, several immunoreactive bands in and around the 34kDa region were present in crude bovine brain extracts. Two major C-terminal immunoreactive bands that comigrated with $p34^{cdc2}$ were resolved. The higher molecular

Figure 1: Epitopic Sites for C-terminal, PSTAIRE and N-terminal Antibodies

Antibodies raised against synthetic peptides derived from three distinct regions of human $p34^{cdc2}$. The N-terminal and PSTAIRE regions are highly conserved regions among $p34^{cdc2}$ homologues while the C-terminal region is more specific for human $p34^{cdc2}$.



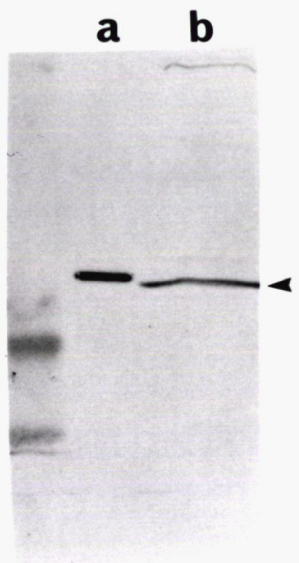
ANTIBODY	SEQUENCE
1. N-terminal	C-E-K-I-G-E-G-T-Y-G-V-V-Y-K
2. PSTAIRE	C-E-G-V-P-S-T-A-I-R-E-I-S-L-L-K-E
3. C-terminal	C-N-D-L-D-N-Q-I-K-K-M

weight band at 35 kDa also appeared to cross react with PSTAIRE antibodies, as a Western blot of crude extracts with both C-terminal and PSTAIRE mixed together did not indicate the presence of a separate immunoreactive protein (Figure 2). In addition to this 35kDa band, several other PSTAIRE immunoreactive proteins were detected, at approximately 45kDa, 30kDa and 28kDa. Only one major band at 33kDa was detected with N-terminal antibody in 100,000xg extracts, although a faint band at 35kDa has also been detected in partially purified extracts (data not shown). The verification of the specificity of these immunoreactive bands was not tested using an excess of competing peptide, as all antibodies used for Western blotting were affinity purified on peptide columns. The possibility that some of these bands are proteolytic degradation products is possible but highly unlikely, as extraction of cytosolic extract was performed in the presence of a battery of protease inhibitors. These data are indicative of the presence of numerous proteins sharing similar epitopic sites to those found in human $p34^{cdc2}$ and suggest that there are related forms that are distinct from the authentic $p34^{cdc2}$ protein in bovine brain extracts.

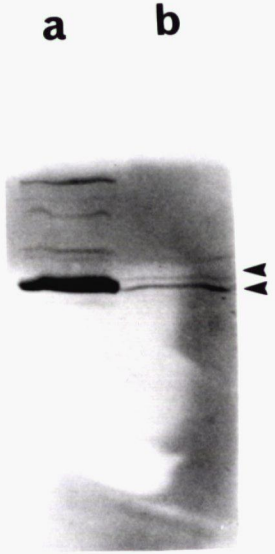
At this particular time, a protocol had been optimized for the purification of a proline directed kinase whose substrate specificity appeared to resemble that of $p34^{cdc2}$, as its activity required a C-terminal proline immediately following the phosphorylatable residue on peptide substrates (Lew *et al.*, 1992a). The protocol for purification of the brain PDK (Lew *et al.*, 1992a) is shown in Figure 3. The cytosolic fraction of bovine brain was loaded on a DEAE-Sepharose column connected directly to an Affigel blue column. Kinase activity did not bind to the DEAE column and activity was eluted from Affigel blue column with 1.5M NaCl. Additional chromatographic steps included Sepharose 4B, hydroxylapatite, Superose 12 and finally Mono S FPLC chromatography. Kinase activity was monitored with the pro-src peptide, a peptide derived from three $p34^{cdc2}$ *in vitro* phosphorylation sites in $pp60^{c\text{-}src}$. To investigate the possibility that one or more of the $p34^{cdc2}$ immunoreactive bands found on Western Blots corresponded to the active proline

Figure 2: Western Blot of Cytosolic Extracts of Bovine Brain with N-terminal, PSTAIRE and C-terminal Antibodies

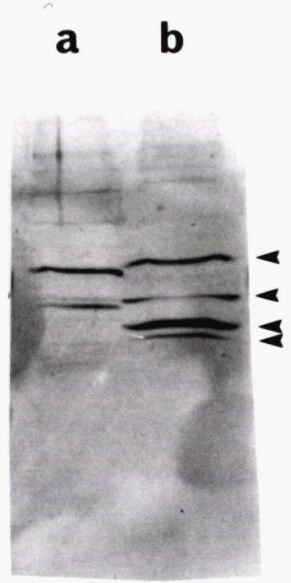
Bovine brain cytosolic extracts and partially purified HeLa cell $p34^{cdc2}$ were prepared as described in the "Experimental Procedures" under purification procedures. Equal amounts of protein were subjected to SDS-PAGE and then transblotted to Immobilon-P as described in the "Experimental Procedures" with HeLa cell control in the lanes marked "a" and the bovine brain cytosolic extracts marked "b" . The membrane was then probed with N-terminal, PSTAIRE or C-terminal primary antibody and alkaline phosphatase-linked secondary antibody. Immunoreactive proteins were detected using BCIP and NBT as color substrates. Immunoreactive proteins are indicated by the arrow markers.



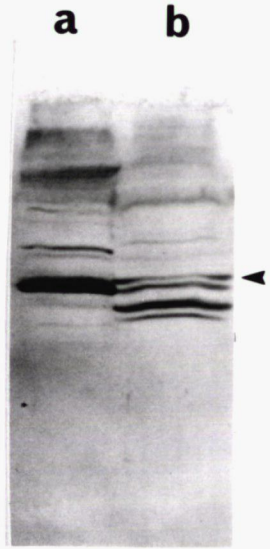
N-ter



C-ter



PSTAIRE



C-ter &
PSTAIRE

directed protein kinase, purified fractions from the Mono-S column were immunoblotted with the three $p34^{cdc2}$ antibodies and proline-directed kinase activity monitored with the pro-src peptide. Fold purification in the peak fraction of kinase activity was estimated to be approximately 200,000 fold (Lew *et al.*, 1992a). As shown in Figure 4A, N-terminal immunoreactivity towards a 33kDa protein was present in Mono-S fractions 27 through 32, with the peak of immunoreactivity occurring in fraction 28. This correlated with both kinase activity towards the pro-src peptide as well as a 33kDa silver stained band on SDS gels (data not shown (Lew *et al.*, 1992a)). Antibodies towards the C-terminal region were unable to detect any immunoreactive proteins in Mono-S fractions (Figure 4C), while antibodies towards the PSTAIRE sequence reacted weakly with the 33kDa N-terminal immunoreactive protein (Figure 4B). The immunoreactive properties of this enzyme indicated that the proline kinase was related to but distinct from authentic $p34^{cdc2}$.

The N-terminal antibody is directed to a region of $p34^{cdc2}$ which contains the triad of glycines present in all protein kinases that is involved in ATP binding (Hanks *et al.*, 1988). It is possible that the N-terminal antibody is not specific for $p34^{cdc2}$ and related family members but can cross react with other protein kinases. To test this possibility, several serine and threonine kinases as well as the tyrosine kinase, $p56^{lyn}$ (Cheng *et al.*, 1992, Litwin *et al.*, 1991), purified from bovine spleen were tested for immunoreactivity towards the N-terminal antibody (Figure 5). Only $p34^{cdc2}$ and the brain PDK displayed N-terminal immunoreactivity when an equal or greater amount of the other protein kinases was used, thus confirming the specificity of this antibody for $p34^{cdc2}$ and related family members. Recently the 33kDa subunit has been cloned and sequenced (Lew *et al.*, 1992b). Table 3 shows the sequences corresponding to peptides used to make the peptide antibodies, HeLa cell $p34^{cdc2}$ and the corresponding sequence regions of the brain PDK from the N-terminal and PSTAIRE regions. The sequence information from the 33kDa

Figure 3: Purification Scheme for the Brain Proline Directed Kinase

Purification Scheme for Brain PDK

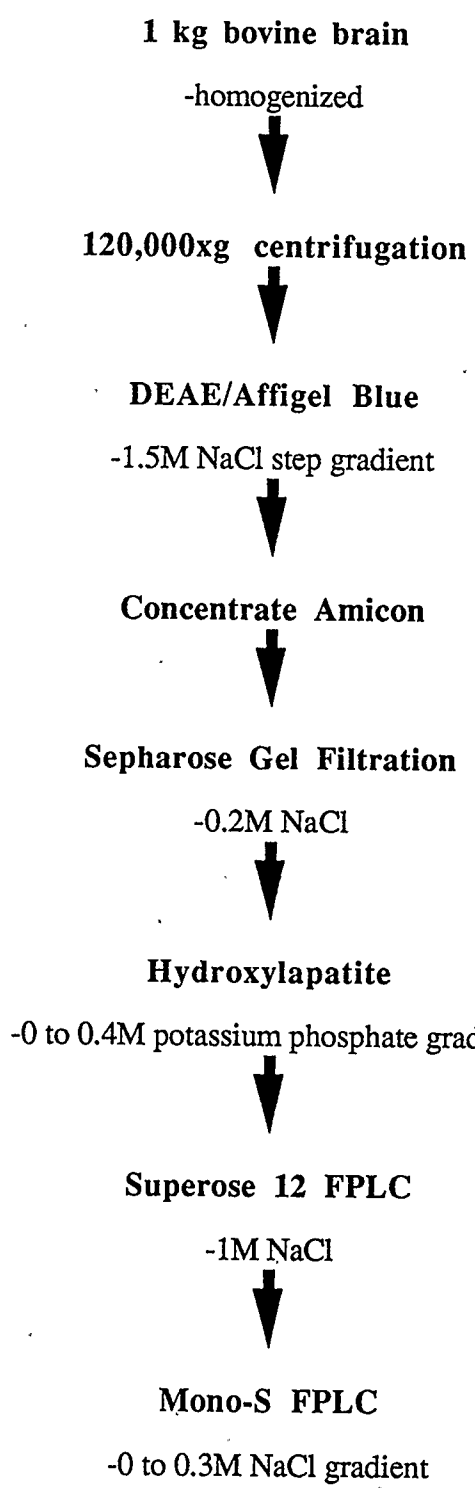
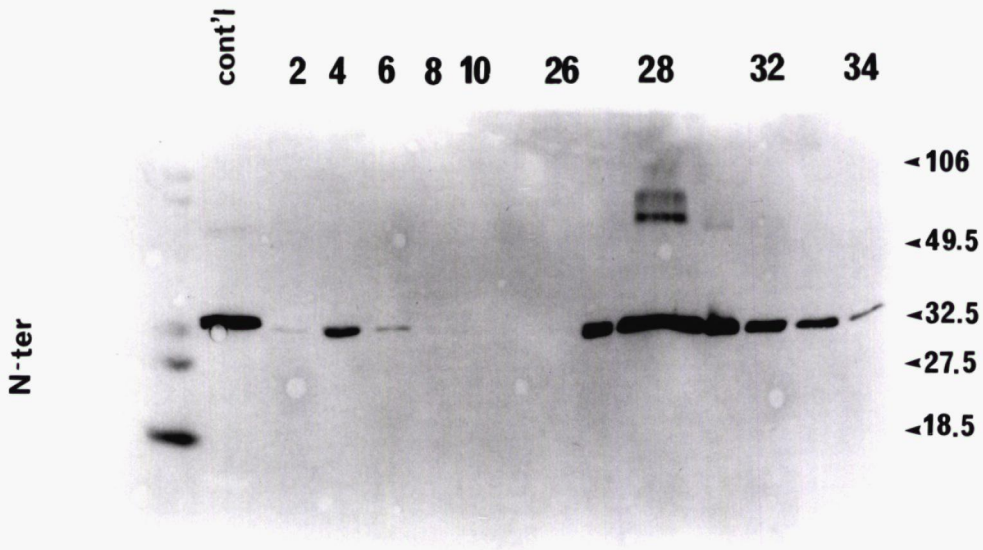
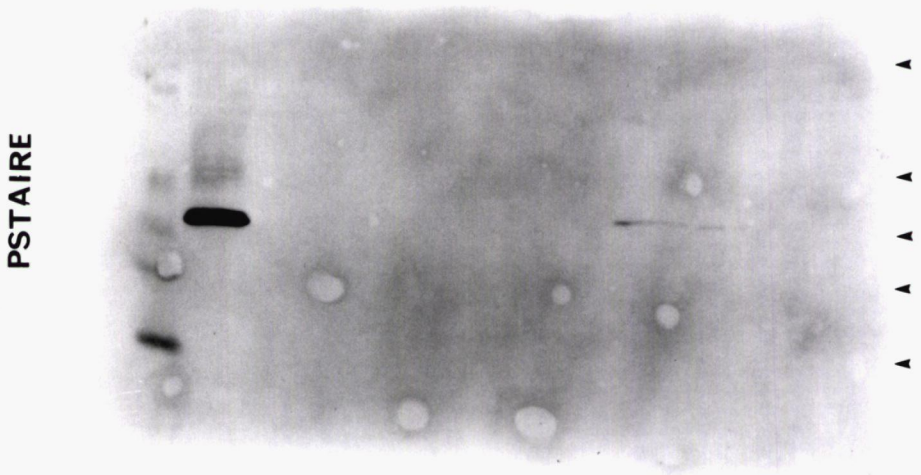


Figure 4: Immunoreactive Properties of Mono-S Fractions Containing the Brain PDK

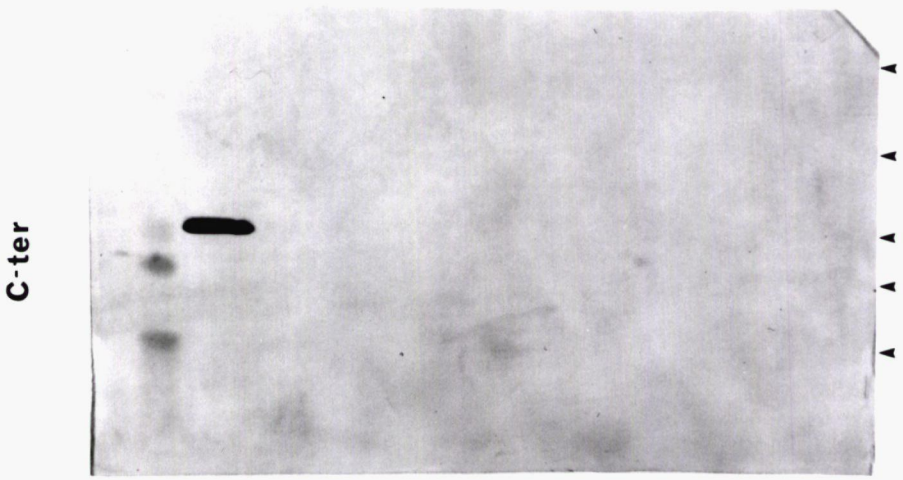
Equal volumes of Mono-S fractions containing the brain PDK were electrophoresed and transblotted onto Immobilon-P membranes as described in the "Experimental Procedures". After incubation with (A) N-terminal, (B) PSTAIRE or (C) C-terminal primary antibody, immunoreactive bands were visualized using an alkaline phosphatase conjugated secondary antibody and NBT/BCIP as substrates.



A



B



C

Figure 5: Specificity of the $p34^{cdc2}$ N-terminal antibody

Purified protein kinases were applied to SDS-polyacrylamide gels and subsequently transblotted to Immobilon-P. Membranes were probed with N-terminal antibody and immunoreactive proteins were detected with alkaline phosphatase conjugated secondary antibody as described in the "Experimental Procedures". Lane 1: HeLa $p34^{cdc2}$; Lane 2: purified brain PDK (0.1μg); Lane 3: protein kinase C (0.5μg); Lane 4: cAMP-dependent protein kinase (0.5μg); Lane 5: phosphorylase kinase (2.0μg); Lane 6: smooth muscle myosin light chain kinase (0.6μg); Lane 7: $p56^{lyn}$ (0.1μg).

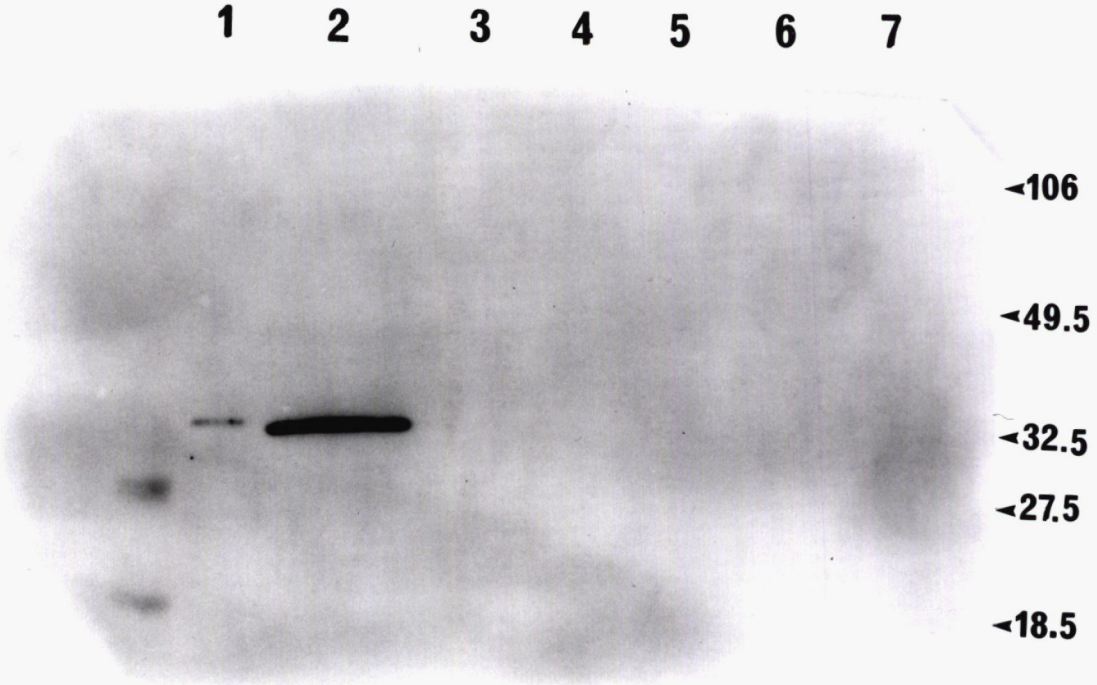

1
2
3
4
5
6
7
106
49.5
32.5
27.5
18.5

Table 3: Sequence Comparison of HeLa cell $p34^{cdc2}$ and the Brain PDK in N-terminal and C-terminal Regions

	N-terminal	PSTAIRE
Antibody	C-E-K-I-G-E-G-T-Y-G-V-V-Y-K	C-E-G-V-P-S-T-A-I-R-E-I-S-L-L-K-E
Brain PDK	E-K-I-G-E-G-T-Y-G-T-V-F-K	E-G-V-P-S-S-A-L-R-E-I-C-L-L-K-E
HeLa $p34^{cdc2}$	E-K-I-G-E-G-T-Y-G-V-V-Y-K	E-G-V-P-S-T-A-I-R-E-I-S-L-L-K-E

subunit of the brain PDK correlates with its immunoreactive properties. The N-terminal sequence has only two amino acid changes in the brain PDK; a threonine replacing a valine residue and phenylalanine replacing a tyrosine residue. Within the corresponding PSTAIRE region of the brain PDK, there are three amino acid substitutions: a serine replacing threonine, leucine replacing isoleucine and cysteine replacing serine, accounting for the weaker immunoreactive properties of the brain PDK towards PSTAIRE antibodies. Corresponding regions of the brain PDK and HeLa cell $p34^{cdc2}$ are completely divergent, accounting for the inability of C-terminal antibodies to recognize the brain PDK.

Substrate Specificity Characterization of the Brain PDK

The establishment of the brain PDK as a member of the cdc2 kinase family and accessibility to reasonable quantities of purified enzyme prompted us to examine some of its biochemical and enzymological properties, in particular its substrate specificity. A consensus motif for $p34^{cdc2}$ phosphorylation, **S/T**-P-X-K/R, has been proposed based on the analysis of *in vitro* and *in vivo* phosphorylation sites of numerous $p34^{cdc2}$ substrates (Moreno and Nurse, 1990). There are several key observations that led us to believe that a similar consensus sequence could exist for the brain PDK. First, the brain PDK is immunologically related to $p34^{cdc2}$ and shows high sequence similarity to other cdc2 family members (Lew *et al.*, 1992b). Second, both $p34^{cdc2}$ and the brain PDK have an absolute requirement for proline immediately C-terminal to the phosphorylatable residue on synthetic peptide substrates (Lew *et al.*, 1992a) and both enzymes phosphorylate caldesmon and neurofilaments H and M on identical sites as determined from tryptic peptide mapping experiments (Lew *et al.*, 1992b, Alan Mak, personal communication).

Synthetic peptides have not yet been used to test the proposed $p34^{cdc2}$ consensus motif. Substrate specificity characterization of the brain PDK using synthetic peptides would therefore represent one of the first studies of its kind for a purified cdc2-family

member. At the same time it would allow for the verification of specific residues within the proposed $p34^{cdc2}$ consensus. Kinetic parameters for brain PDK-catalyzed phosphorylation of three peptides, bradykinin, the pro-src peptide, and a histone-derived peptide, HS(1-18), which contained the repeat phosphorylation site for the growth associated histone H1 kinase (Matthews and Huebner, 1984), are shown in Table 4. Representative plots of initial rate versus substrate concentration and their corresponding Lineweaver-Burke plots are shown in Figure 6 (bradykinin), Figure 7 (pro-src) and Figure 8 (HS(1-18)). All three peptides contained the proposed consensus sequence for phosphorylation by $p34^{cdc2}$; however, marked differences in kinetic parameters for the three peptide substrates were observed. In agreement with previous data (Lew *et al.*, 1992a), the histone peptide HS(1-18) was a much better substrate having an apparent K_m value of 5μM, well over two orders of magnitude lower than those values for pro-src or bradykinin peptides, and a relative V_{max} two to three fold higher than either peptide. These differences in kinetic parameters, particularly in the K_m values, suggested that there might be other important determinants in addition to what was contained in the proposed motif for $p34^{cdc2}$ phosphorylation. The K_m value for native histone was slightly higher than the 18-mer peptide at 12μM, while the relative V_{max} was almost identical (Table 4 and Figure 9). The comparable kinetic parameters for native histone H1 and the histone peptide suggested that determinants required for efficient phosphorylation of the intact protein were also contained within the histone peptide sequence.

Histone proteins are commonly used as *in vitro* substrates not only for $p34^{cdc2}$, but also for other kinases such as cAMP dependent protein kinase, protein kinase C and CaM kinase II. The ability of these enzymes to phosphorylate the full length histone peptide was tested in order to address the specificity of this peptide for $p34^{cdc2}$ and related kinases. Phosphate incorporation into the full length histone peptide HS(1-18) was negligible for all three enzymes (J. Lew, personal communication). In addition, synthetic peptides designed

Table 4: Kinetic Parameters of Synthetic Peptide Substrates and Native Histone H1 for the Brain PDK

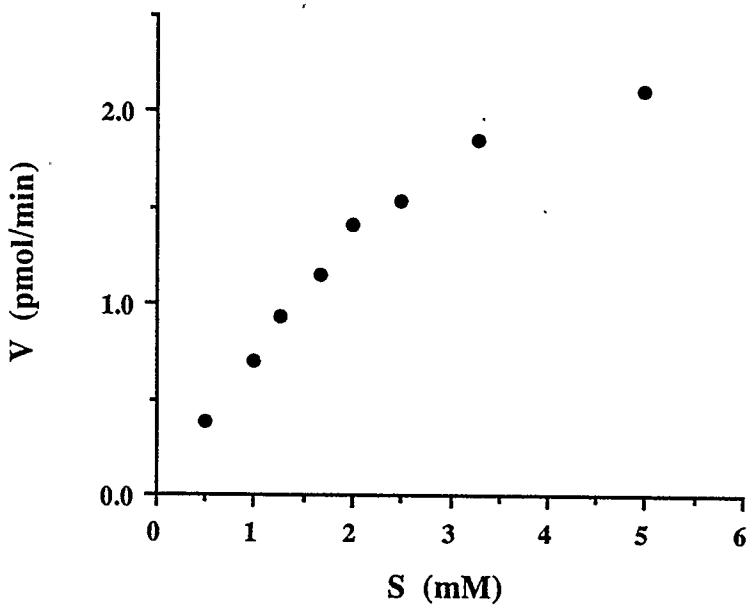
Kinetic parameters were determined as detailed under "Experimental Procedures". The relative maximal velocity of the phosphorylation reaction, in pmols/min/µl of enzyme, was expressed as a percentage of the full length histone peptide (HS(1-18)). V_{max}/K_m was also expressed as a percent of the full length peptide.

Peptide	Sequence	K_m (µM)	V_{max} % control	V_{max}/K_m % control
HS(1-18)	K-T-P-K-K-A-K-K-P-K-T-P-K-K-A-K-K-L	5	100	100
Bradykinin	P-P-G-F-S-P-F-R	4200	56	<0.1
Pro-src	R-R-P-D-A-H-R-T-P-N-R-A-F	1700	30	<0.1
Histone		12	105	70

Figure 6: Kinetic Plots for Bradykinin Phosphorylation

A representative plot of the effect of substrate concentration on the rate of phosphorylation of bradykinin for the brain PDK is shown in panel A, with the corresponding Lineweaver-Burke plot shown in panel B. Kinetic parameters were determined as described in the "Experimental Procedures".

A



B

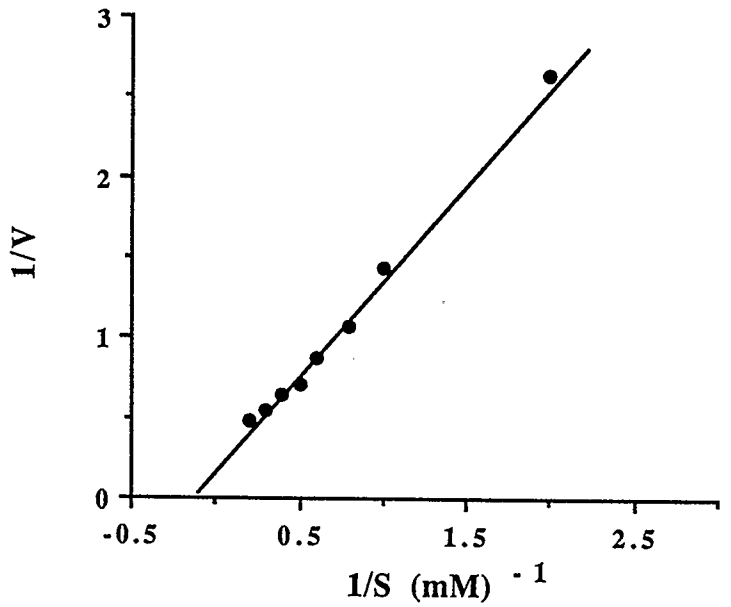
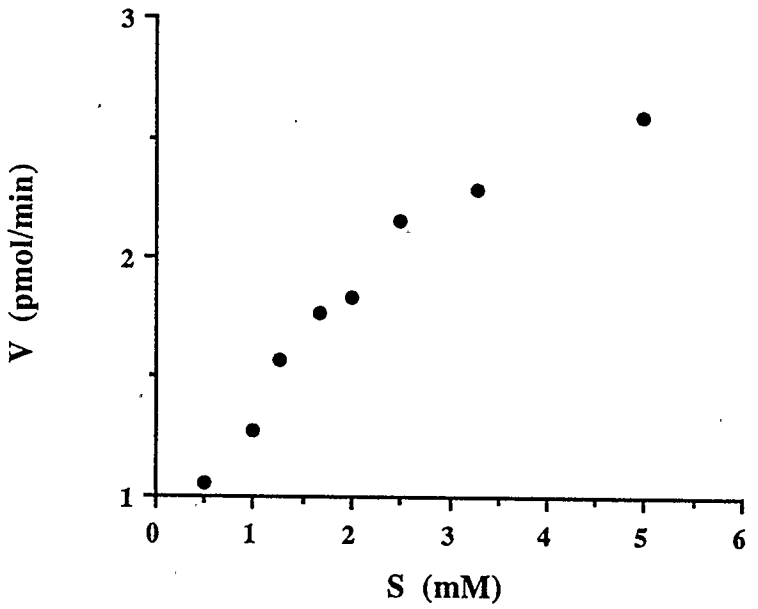


Figure 7: Kinetic Plots for Pro-src Phosphorylation

A representative plot of the effect of substrate concentration on the rate of phosphorylation of pro-src peptide for the brain PDK is shown in panel A, with the corresponding Lineweaver-Burke plot shown in panel B. Kinetic parameters were determined as described in the "Experimental Procedures".

A



B

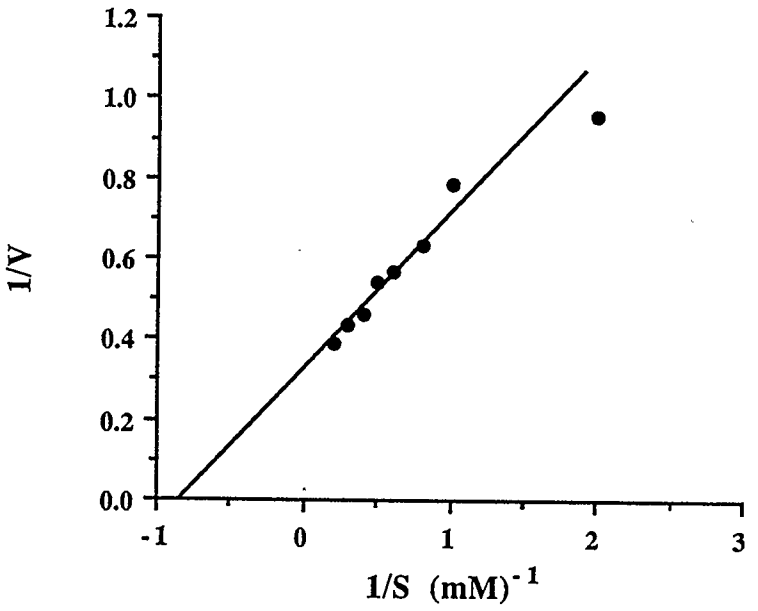
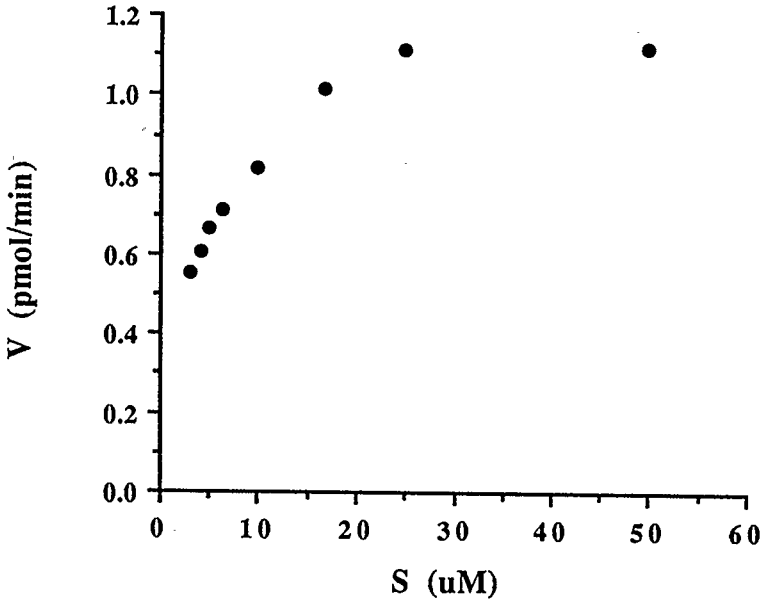


Figure 8: Kinetic Plots for HS(1-18) Phosphorylation

A representative plot of the effect of substrate concentration on the rate of phosphorylation of the full length histone peptide HS(1-18) for the brain PDK is shown in panel A, with the corresponding Lineweaver-Burke plot shown in panel B. Kinetic parameters were determined as described in the "Experimental Procedures".

A


1.2
1.0
0.8
0.6
0.4
0.2
0.0
V (pmol/min)
0
10
20
30
40
50
60
S (uM)

B

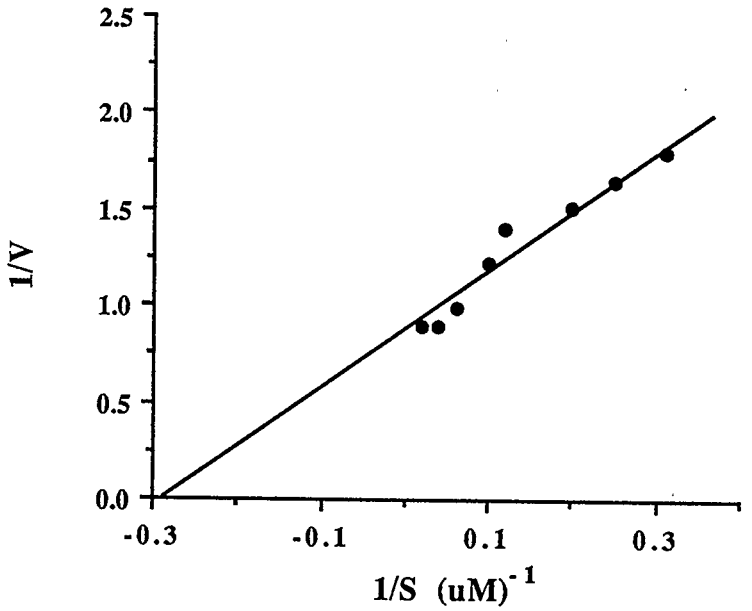
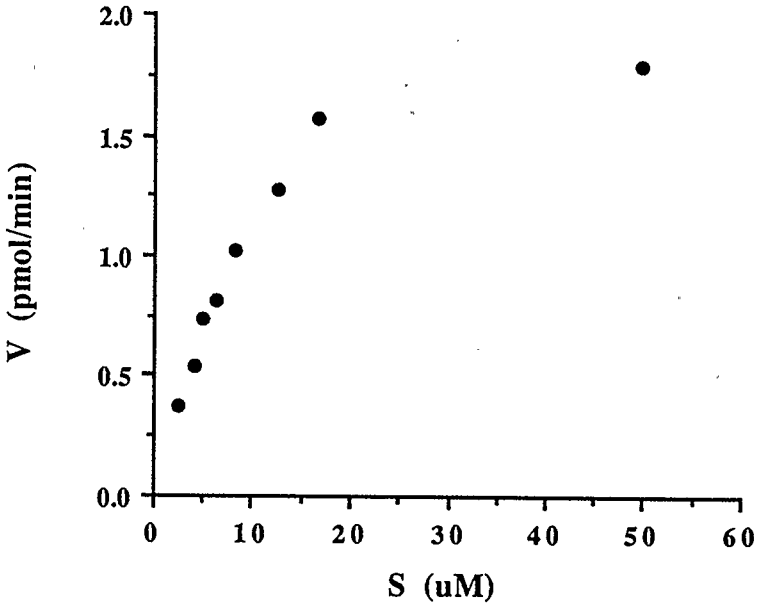

2.5
2.0
1.5
1.0
0.5
0.0
1/V
-0.3
-0.1
0.1
0.3
1/S (uM)-1

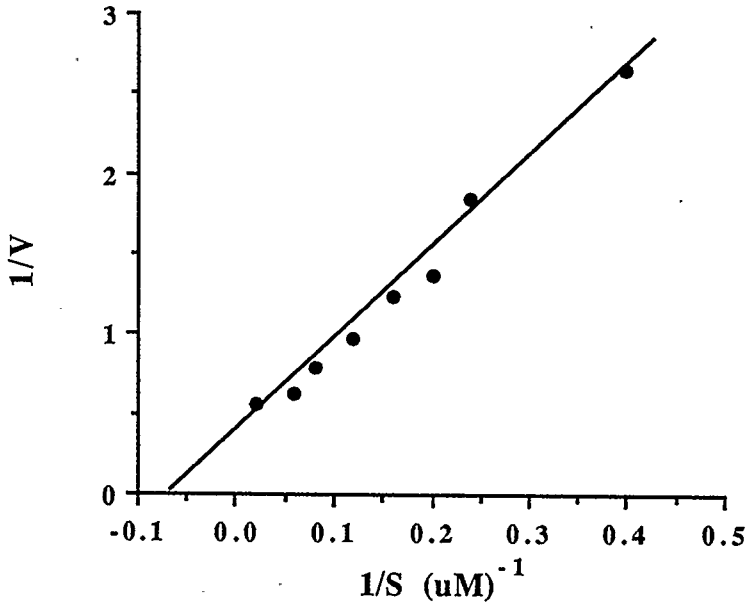
Figure 9: Kinetic Plots for Histone H1 Phosphorylation

A representative plot of the effect of substrate concentration on the rate of phosphorylation of winter flounder histone H1 for the Brain PDK is shown in panel A, with the corresponding Lineweaver-Burke plot shown in panel B. Kinetic parameters were determined as described in the "Experimental Procedures".

A


2.0
1.5
1.0
0.5
0.0
V (pmol/min)
0
10
20
30
40
50
60
S (uM)

B


3
2
1
0
1/V
-0.1
0.0
0.1
0.2
0.3
0.4
0.5
1/S (uM)$^{-1}$

as specific substrates for other protein kinases, including Kemptide, MLCK substrate peptide and several protein kinase C pseudosubstrate peptides, were negligibly phosphorylated by both $p34^{cdc2}$ and the brain PDK (Table 5). Thus the histone peptide appears to be a specific and unique peptide substrate of cdc2-like protein kinases.

The full length histone peptide (HS(1-18)) contains two possible $p34^{cdc2}$ consensus phosphorylation sites. Stoichiometry of phosphorylation revealed that both sites were phosphorylated by the brain PDK (Table 6). If substrate concentration is expressed on the basis of phosphorylation site rather than peptide, the K_m value is therefore 10μM of phosphorylation sites. Kinetic parameters for the full length histone peptide HS(1-18) and the corresponding peptide having the first phosphorylatable residue replaced with alanine are shown in Table 7. Substitution of the first phosphorylatable residue with an alanine (HS(1-18)A2), resulted in a small K_m increase, to 15μM, and also a drop in relative V_{max} (Table 7). These results suggest that the two phosphorylation sites in the histone peptide are independently phosphorylated with similar kinetics of phosphorylation. This is in contrast to the previous observation that the phosphorylation of two proximal sites (separated by 5 amino acid residues) in caldesmon by the HeLa $p34^{cdc2}$ kinase display strong negative co-operativity (Mak *et al.*, 1991a).

The amino acid sequence around the two phosphorylation sites of the histone peptide HS(1-18) differ mainly in the amino terminal regions of the target residues. While the threonine residue at position 2 of the full length peptide is preceded by a single lysine residue, a number of lysine residues forming a positive charge cluster are present in the amino terminal region of the threonine residue at position 11. The contribution of these lysine residues to the peptide's substrate activity was examined using truncated peptide analogues with successive deletions of the lysine residues. Table 7 shows that the substrate activities of truncated peptides HS(4-18) and HS(9-18) are not markedly different from that of HS(1-18), indicating that basic clusters N-terminal to the phosphorylatable

Table 5: Relative Rates of Phosphorylation of Various Peptide Substrates by Brain PDK and HeLa cell $p34^{cdc2}$

Kinase reactions were performed as described in the "Experimental Procedures" in the presence of 5 mM HS(9-18) peptide, MLCK pseudosubstrate peptide, PKC ε and ζ peptides, based on their respective pseudosubstrate domains, and a peptide derived from myelin basic protein. Results are expressed as a percent of ^{32}P incorporation into HS(9-18).

Peptide Substrate	Brain PDK	HeLa $p34^{cdc2}$
	% ^{32}P-incorporation	
HS(9-18) P-K-T-P-K-K-A-K-K-L	100	100
Kemptide L-R-R-A-S-L-G	0.1	0.1
MLCK Peptide K-K-R-A-A-R-A-T-S-N-V-F	4.9	0
PKC Peptides		
E-R-M-R-P-R-K-R-Q-G-S-V-R-R-R-V (PKC-ε)	6.8	9.5
G-E-D-K-S-I-Y-R-R-G-S-R-R-W-R-K-L (PKC-ζ)	0	6.3
A-Q-K-R-P-S-Q-R-S-K-Y-L (MBP)	0	6.3

Table 6: Stoichiometry of Phosphorylation of Histone Peptides

Phosphorylation reactions were performed as described in the "Experimental Procedures". Values shown represent the average of three separate experiments, after incubation for 60 minutes.

Peptide	Sequence	mole PO_4^- / mole peptide
HS(1-18)	K-T-P-K-K-A-K-K-P-K-T-P-K-K-A-K-K-L	1.86
HS(1-18)A2	K-A-P-K-K-A-K-K-P-K-T-P-K-K-A-K-K-L	0.88
HS(4-18)	K-K-A-K-K-P-K-T-P-K-K-A-K-K-L	0.81
HS(9-18)	P-K-T-P-K-K-A-K-K-L	1.07

Table 7: Influence of N-terminal Truncations on Kinetic Parameters for the Brain PDK

Kinetic parameters were determined as detailed under "Experimental Procedures". The relative maximal velocity of the phosphorylation reaction, in pmols/min/µl of enzyme, was expressed as a percentage of the full length histone peptide (HS(1-18)). V_{max}/K_m was also expressed as a percent of the full length peptide.

Peptide	Sequence	K_m (µM)	V_{max} % control	V_{max}/K_m % control
HS(1-18)	K-T-P-K-K-A-K-K-P-K-T-P-K-K-A-K-K-L	5	100	100
HS(1-18)A2	K-A-P-K-K-A-K-K-P-K-T-P-K-K-A-K-K-L	15	66	24
HS(4-18)	K-K-A-K-K-P-K-T-P-K-K-A-K-K-L	14	69	52
HS(9-18)	P-K-T-P-K-K-A-K-K-L	6	82	70

residue were not important for efficient substrate phosphorylation by the brain PDK. On the other hand, deletion of the amino terminal proline residue from the peptide HS(9-18) resulted in a peptide, HS(10-18) of markedly lower substrate activity (Table 8). Based on these results, it was concluded that HS(9-18) represented the minimal size peptide substrate that contained all of the important determinants for the brain PDK.

HS(9-18) was chosen as the parent peptide to investigate the effect of single amino acid changes or deletions either N-terminal to the phosphorylatable residue, within the proposed $p34^{cdc2}$ consensus sequence or C-terminal to the consensus sequence, on the ability of the brain PDK to phosphorylate these peptide substrates. There are two residues, a lysine and a proline that are N-terminal to the phosphorylatable residue. The proline at the -2 position is a determinant for the MAP kinases (Clark-Lewis *et al.*, 1991, Gonzalez *et al.*, 1991) and also appears to contribute to efficient substrate phosphorylation by the brain PDK. This observation was not due to contamination of the enzyme preparation with a MAP kinase, as Western blots of the purified enzyme preparation with anti-MAP kinase antibodies shows no immunoreactivity (data not shown). Substitution of this proline residue for an alanine ((HS(9-18)A9), or deletion of this residue resulted in approximately a 4 or 10-fold increase in K_m values respectively (Table 8). Further deletion of the lysine residue resulted in a peptide, HS(11-18), showing an additional 10-fold increase in K_m value. The effect of this lysine deletion could effectively be reversed if the α-amino group of the threonine residue was acetylated (Table 8). The result suggests that the lysine residue at the -1 position does not contribute directly as a positive determinant, but neutralizes the strong negative influence of the free α-amino group of the target threonine residue. This suggestion is supported by the observation that substitution of the lysine residue at the -1 position by alanine (HS(9-18)A10) does not significantly change the substrate activity of the peptide (Table 8).

Table 8: Influence of N-terminal Substitutions and Deletions on Kinetic Parameters for the Brain PDK

Kinetic parameters were determined as detailed under "Experimental Procedures". The relative maximal velocity of the phosphorylation reaction, in pmols/min/μl of enzyme, was expressed as a percentage of the parent peptide (HS(9-18)). V_{max}/K_m was also expressed as a percent of the parent peptide.

Peptide	Sequence	Km (μM)	Vmax % control	Vmax/Km % control
HS(9-18)	P-K-T-P-K-K-A-K-K-L	6	100	100
HS(9-18)A9	A-K-T-P-K-K-A-K-K-L	24	95	49
HS(9-18)A10	P-A-T-P-K-K-A-K-K-L	4	107	145
HS(10-18)	K-T-P-K-K-A-K-K-L	57	178	19
HS(11-18)	T-P-K-K-A-K-K-L	500	72	<1
HS(Ac11-18)	Acetyl-T-P-K-K-A-K-K-L	24	137	35

The synthetic peptide approach is especially useful in the testing of proposed sequence motifs for substrate activity of protein kinases. An earlier study has already demonstrated the dependence on proline residues at the +1 position for the substrate activity of peptides pro-src and bradykinin (Lew *et al.*, 1992a). Similarly, when proline 12 of HS(9-18) is substituted with an alanine, the peptide HS(9-18)A12 is essentially inactive as a substrate for the brain PDK. The importance of a positively charged residue at position +3, another feature of the motif, is also verified using synthetic peptides. Substitution of lysine 14 by alanine results in over a 60-fold increase in K_m value of the peptide, and a two fold increase in V_{max} (Table 9). Some serine/threonine kinases are known to have a preference for the amino acid residues they phosphorylate. For example, casein kinase II prefers serine over threonine as the phosphoacceptor, as synthetic peptides containing a serine had a 10-fold higher V_{max} and 20-fold lower K_m value than the equivalent threonine containing peptide (Kemp, 1990). In addition, there is recent evidence for the existence of dual specificity protein kinases, those able to phosphorylate serine, threonine and tyrosine residues (Lindberg *et al.*, 1992). The ability of the brain PDK to phosphorylate histone-derived peptides containing serine, threonine or tyrosine was tested. The brain PDK displayed no significant preference for serine or threonine residues (Table 9), and peptide analogues having tyrosine substituted for threonine in HS(9-18) had no substrate activity .

It has been proposed that a polar residue at the +2 position of the consensus motif for $p34^{cdc2}$ is favored for efficient substrate phosphorylation (Moreno and Nurse, 1990). When the lysine at the +2 position of the parent peptide HS(9-18) is changed to an alanine, the K_m value for HS(9-18)A13 increases almost 10-fold from 6μM to 50μM for the brain PDK with a 60% increase in V_{max} (Table 9). If the same amino acid is substituted by a negatively charged residue (HS(9-18)D13), the K_m value increases over 100 fold relative to the parent peptide and V_{max} values decrease to less than 50% of the parent peptide

Table 9: Influence of C-terminal Residues and Proposed Consensus Sequence Amino Acid Residues on Kinetic Parameters for the Brain PDK

Kinetic parameters were determined as detailed under "Experimental Procedures". The relative maximal velocity of the phosphorylation reaction, in pmols/min/µl of enzyme, was expressed as a percentage of the parent peptide (HS(9-18)). V_{max}/K_m was also expressed as a percent of the parent peptide.

Peptide	Sequence	Km (µM)	Vmax % control	Vmax/Km % control
HS(9-18)	P-K-T-P-K-K-A-K-K-L	6	100	100
HS(9-18)S11	-----S----------	7	243	199
HS(9-18)A14	-----T------A----------	376	187	3
HS(9-18)A13	-----T-----A----------	50	165	20
HS(9-18)D13	-----T-----D----------	649	45	<1
HS(9-18)A16	-----T----------A------	26	140	33
HS(9-18)A17	-----T-----------A-----	27	152	34
HS(9-18)A16,A17	-----T---------A-A-----	155	132	5

(Table 9). Clearly a positively charged amino acid is greatly favored over a negatively charged residue at the +2 position for the brain PDK.

The presence of basic residues in the proposed $p34^{cdc2}$ consensus motif is clearly important for efficient substrate phosphorylation by the brain PDK. To investigate the significance of the C-terminal basic cluster in HS(9-18), lysine residues at +5 and +6 positions relative to the phosphorylatable residue were substituted by alanine. Substitution of either lysine residue, HS(9-18)A16 or HS(9-18)A17, results in approximately a 5-fold increase in K_m values (Table 9), along with a 50% increase in V_{max}. Substitution of both lysine residues, (HS(9-18)A16,A17), causes a further increase in K_m to a value 25 fold higher than that of the parent peptide (Table 9), thus establishing the importance of basic residues downstream from the consensus sequence for efficient substrate binding.

Substrate specificity determinants using synthetic peptides have not been elucidated for various forms of $p34^{cdc2}$. The protocol used routinely for partial purification of $p34^{cdc2}$ from colcemid treated HeLa cells involves $p13^{suc1}$ affinity chromatography followed by hydroxylapatite chromatography. The exact composition of this preparation pertaining to the specific forms of $p34^{cdc2}$ present (ie. associated cyclins and/or phosphorylation states) has not been characterized. Given this fact, in addition to the observation that other cdc2 homologues (cdk2 and cdk3) can bind to p13-Sepharose (Meyerson *et al.*, 1992), kinetic parameters for the partially purified "$p34^{cdc2}$" kinase activity were not deduced. Alternatively, initial rates of phosphorylation of the various peptide analogues at a single peptide concentration were compared. Although not as informative as K_m and V_{max} values, relative initial rates of phosphorylation can lend some insight into $p34^{cdc2}$ specificity determinants. The peptide analogues can be useful as analytical tools to investigate cdc2-kinase substrate specificity as the various forms become purified in future. Figure 10 represents initial rates of phosphorylation for both the brain PDK and partially purified $p34^{cdc2}$ with histone peptides having amino acid substitutions within the proposed

S/T-P-X-K/R motif. Relative rates of phosphorylation are expressed as a percentage of the parent peptide HS(9-18). As expected, $p34^{cdc2}$ kinase activity was proline dependent and required the presence of a basic residue in the +3 position of substrate peptides for favorable phosphorylation (Figure 10). As is the case with the brain PDK, a basic amino acid residue at the +2 position is clearly favored over an acidic or neutral residue, as relative rates of phosphorylation for $p34^{cdc2}$ with peptides having an aspartic acid residue or alanine in place of lysine were 10% and 50% of the parent peptide respectively (Figure 10).

In addressing the role of basic residues outside the proposed consensus motif, $p34^{cdc2}$ and the brain PDK appear to share similar requirements for a basic cluster downstream from the phosphorylation site. These effects however are not as drastic as those described for the brain PDK (Figure 11). Substitution of a lysine residue in HS(9-18) at the +5 or +6 position results in a drop of relative rates of phosphorylation to approximately 60% of the parent peptide. Substitution of both residues simultaneously drops the relative rates of phosphorylation to approximately 25% of the parent peptide. Substitution of the lysine residue immediately N-terminal to the phosphorylatable threonine in HS(9-18) results in comparable rates of phosphorylation for $p34^{cdc2}$ and the brain PDK (Figure 11).

Pseudosubstrate peptides, those having phosphorylatable amino acids replaced with an alanine or another non-phosphorylatable residue, have been reported to be adequate inhibitors of their respective protein kinases (Kemp, 1990). With the brain PDK, the majority of peptide analogues showed altered K_m values while V_{max} values were relatively constant, suggesting that these peptide analogues could not be used as inhibitory peptides. Histone peptide derivatives having the phosphorylatable threonine replaced with an alanine or tyrosine residue (HS(9-18)A11 or HS(9-18)Y11), or a peptide having the proline at the +1 position substituted by an alanine (HS(9-18)A12) were all very poor inhibitors for both

Figure 10: Relative Rates of Phosphorylation by the Brain PDK and HeLa cell $p34^{cdc2}$ for Histone Peptide Derivatives Having Changes in the Proposed $p34^{cdc2}$ Consensus Sequence

The phosphorylation of synthetic peptides (5μM) was performed with purified brain proline directed kinase and partially purified HeLa $p34^{cdc2}$ as described in the "Experimental Procedures". Rates of phosphorylation are expressed as a percentage of parent peptide HS(9-18). Single amino acid substitutions were made by replacing individual residues as indicated. Black bars represent phosphorylation reactions with brain PDK and hatched bars represent reactions with $p34^{cdc2}$.

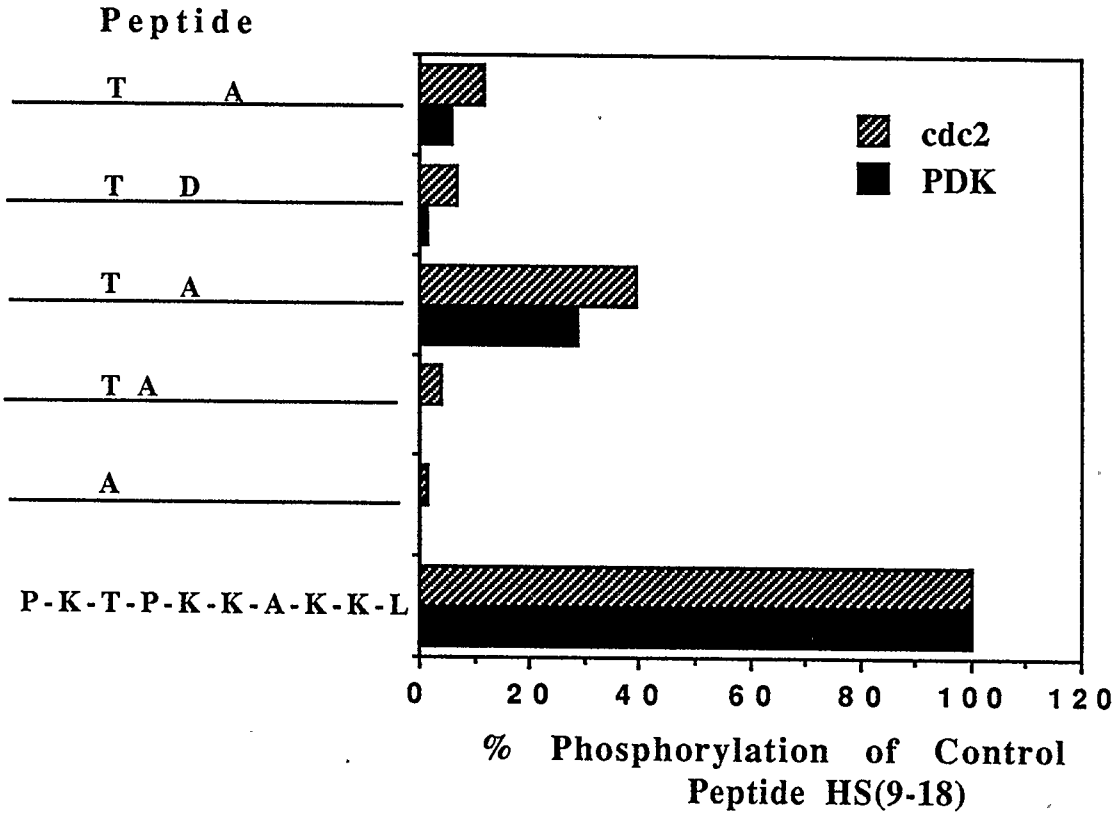
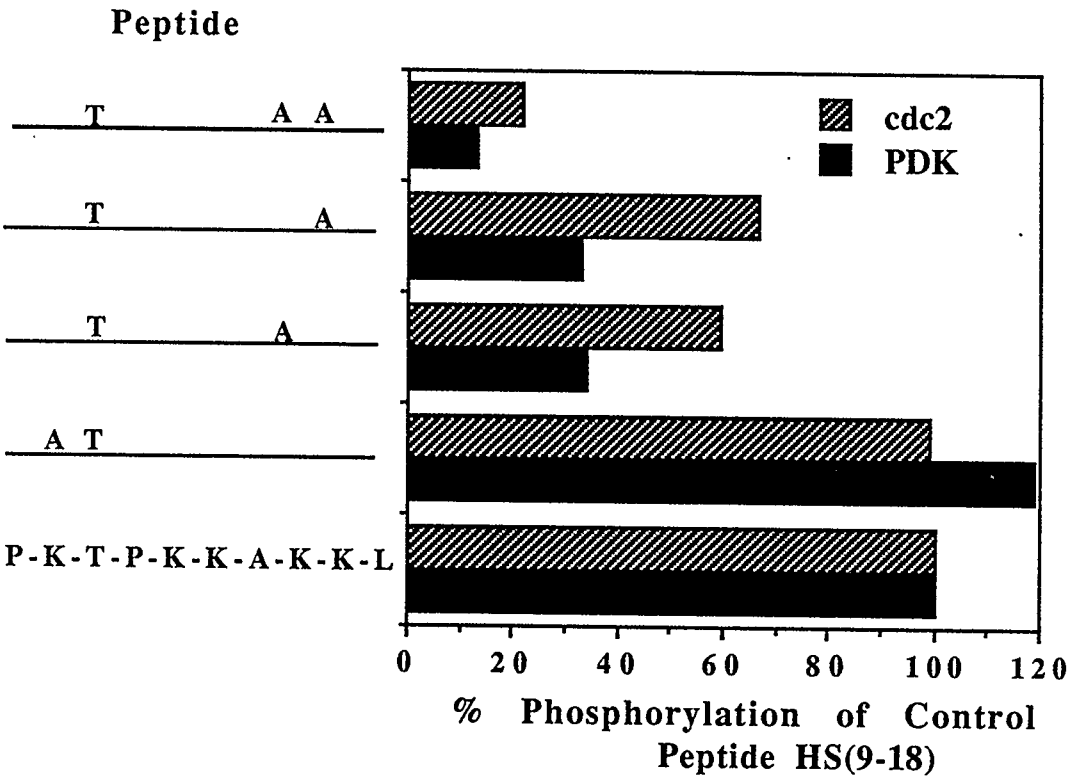

Peptide
T A
T D
T A
T A
A
P-K-T-P-K-K-A-K-K-L
cdc2
PDK
0
20
40
60
80
100
120
% Phosphorylation of Control Peptide HS(9-18)

Figure 11: Relative Rates of Phosphorylation by the Brain PDK and HeLa cell $p34^{cdc2}$ for Histone Peptide Derivatives Having Basic Amino Acid Residues Substituted.

The phosphorylation of synthetic peptides (5μM) was performed with purified brain proline directed kinase or partially purified HeLa cell $p34^{cdc2}$ as described in the "Experimental Procedures". Rates of phosphorylation are expressed as a percentage of the parent peptide HS(9-18). Black bars represent phosphorylation reactions performed with purified brain PDK and hatched bars represent phosphorylation reactions performed with HeLa cell $p34^{cdc2}$.

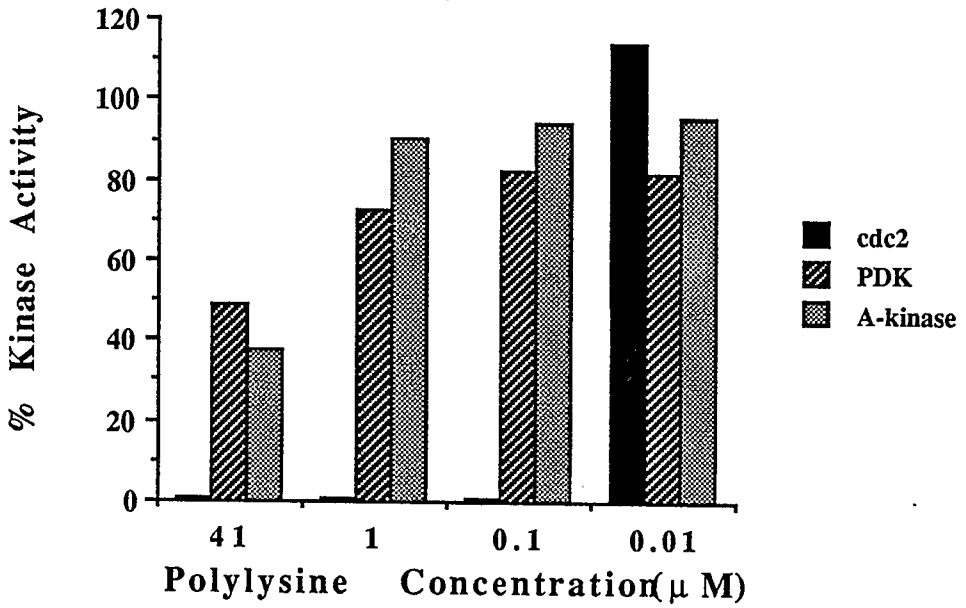

Peptide
T A A
T A
T A
A T
P-K-T-P-K-K-A-K-K-L
cdc2
PDK
0
20
40
60
80
100
120
% Phosphorylation of Control Peptide HS(9-18)

$p34^{cdc2}$ and brain PDK activity. Peptide concentrations up to 10 times the K_m value for HS(9-18) had no effect on $p34^{cdc2}$ or PDK activity (data not shown).

The strong contribution of the basic residues in the histone peptide towards efficient phosphorylation by both the brain PDK and $p34^{cdc2}$ inspired us to perform an initial characterization of the effect of polycations on $p34^{cdc2}$ and the brain PDK-catalyzed phosphorylation of HS(9-18). The effects of polylysine on cAMP-dependent protein kinase-catalyzed phosphorylation of Kemptide was included to represent a kinase unrelated to the cdc2-family. Preliminary experiments indicated that polylysine completely inhibited $p34^{cdc2}$-catalyzed phosphorylation of HS(9-18) at 100nM, while this inhibitory effect was not seen at 10nM. Closer examination of the effects of polylysine revealed that 50% inhibition occured at approximately 30nM. The inhibitory effects of polylysine on brain PDK kinase activity were markedly different, as only 50% of its activity was inhibited at 40mM. This resembled the inhibition of cAMP-catalyzed phosphorylation of Kemptide (Figure 12). The ability of polylysine to inhibit $p34^{cdc2}$ kinase activity did not appear to depend on the substrate used as similar effects were observed when native histone H1 was used as a substrate. The inhibitory potency of polylysine towards $p34^{cdc2}$ kinase activity did however vary depending on the preparation of $p34^{cdc2}$. In a separate experiment, inhibitory activity of polylysine was tested on two other preparations of HeLa cell $p34^{cdc2}$. For the first preparation, complete inhibition of cdc2 kinase activity was observed at a polylysine concentration of 1mM. The second preparation showed 50% inhibition at 100nM. These observations, although preliminary, are interesting given the importance of basic residues for efficient phosphorylation of synthetic peptides by the brain PDK. A more careful examination of the effects of polycations is required to substantiate these data and lend further insight into their effects on cdc2-family members.

Figure 12: The Effect of Polylysine on $p34^{cdc2}$, PDK and cAMP-dependent Kinase Activities

Phosphorylation reactions were carried out as described in the "Experimental Procedures" using 5μM HS(9-18) as a peptide substrate for $p34^{cdc2}$ and the brain PDK and Kemptide as a substrate for the cAMP-dependent protein kinase. Polylysine (24 kDa) was included at the concentrations indicated.


% Kinase Activity
120
100
80
60
40
20
0
41
1
0.1
0.01
Polylysine Concentration(μ M)
cdc2
PDK
A-kinase

DISCUSSION

A clearer understanding of the function and regulation of $p34^{cdc2}$ within the cell cycle is now only beginning to surface. As researchers have delineated the many complex aspects of this process, they have uncovered a growing family of related enzymes whose function and regulatory properties remain unclear. The existence of these related family members, and their potential to possess functions that are outside of cell cycle control, is an interesting prospect that merits further study. Techniques employing molecular biology and yeast genetics have been predominant in the attempts of researchers to understand the overall role of these enzyme species, while the biochemical and enzymological characterization of these kinases, that is more suited for the elucidation of their mechanisms of action, has been limited. It is our hope that this latter approach might result in many interesting observations that further our understanding of the processes controlling cell proliferation and regulation. The present study has focussed on the immunological and substrate specificity characterization of a purified cdc2-related kinase from bovine brain for this purpose.

The identification of a cdc2-related protein in brain was first reported by Draetta and colleagues, who suggested that this protein, because it was present in such a highly non-proliferative tissue, might function in a process unrelated to the cell cycle (Draetta *et al.*, 1988). Using cytosolic extracts prepared from various rat organs, they detected a 34kDa protein that cross-reacted with polyclonal antibodies made against the yeast cdc2 protein, and found high levels of this protein in brain cytosolic extracts. In addition Draetta and his colleagues showed that PC12 cells, a cell line that undergoes differentiation to a neuronal like state in response to nerve growth factor, showed no difference in the levels of this $p34^{cdc2}$-related protein in growth factor treated or untreated cells (Draetta *et al.*, 1988). Since this initial discovery, the existence of $p34^{cdc2}$ or $p34^{cdc2}$-like proteins in brain has

revealed some controversial results. In contrast to Draetta's results, Hasimoto and Kishimoto reported as unpublished results in a recent paper that a monoclonal PSTAIR antibody and cDNA of mouse cdc2 failed to detect cdc2 protein or mRNA of cdc2, respectively, in adult mouse brain (Hisanaga *et al.*, 1991). This was substantiated in another study using Northern blot analysis of neuronal precursor cells that revealed that cdc2 mRNA was dramatically downregulated upon terminal differentiation of neurons (Hayes *et al.*, 1991). More recently, however, M. Hellmich and his colleagues have cloned a cdc2-like kinase from a rat brain cDNA library that is 58% identical to mouse cdc2 and is localized at high levels in terminally differentiated neurons (Hellmich *et al.*, 1992). In addition to these observations, they supported previous findings in Northern blots that very low levels of cdc2 and cdk2 existed in adult rat brain. The apparent discrepancy of the presence of $p34^{cdc2}$ in brain can be reconciled by the obvious existence of related cdc2 family members. Clearly there are other cdc2 homologues whose sequence differences within certain regions account for the inability of these forms to react with antibodies or to recognize cDNA probes derived from authentic yeast cdc2. On the other hand, the lack of specificity of certain cdc2 antibodies allows them to react with multiple cdc2 family members.

Western blots of bovine brain cytosolic extracts, using peptide antibodies derived from three separate regions of $p34^{cdc2}$ (two of them being highly conserved among cdc2 family members), substantiates the existence of several $p34^{cdc2}$-related proteins in this tissue. Immunological characterization of the brain PDK using three polyclonal antibodies derived from human $p34^{cdc2}$ was instrumental in establishing it as a distinct cdc2-family member. Brain PDK, resolved on a Mono-S FPLC column in the final stage of purification, comigrated with a single band possessing very strong N-terminal immunoreactivity, weaker PSTAIRE immunoreactivity and no immunoreactivity towards C-terminal antibodies. Several other cdc2-related proteins present in crude bovine brain

extracts react quite strongly with PSTAIRE antibodies. This in itself is interesting given that this sequence is highly conserved among functional cdc2-homologues and that authentic cdc2 and cdk2 are present in low amounts in brain. Several of these proteins are also of higher molecular weight than HeLa cell $p34^{cdc2}$ suggesting that they possess extensions in sequence beyond what constitutes the catalytic domain for $p34^{cdc2}$. These extensions might prove to be important regulatory domains for protein/protein interaction or for targeting the enzyme to a particular location within the cell. This would not be unlikely, as a 58kDa cdc2 homologue cloned by Bunnell *et al.* contains what corresponds to a calmodulin binding domain (Bunnell *et al.*, 1990). With the exception of the 33kDa immunoreactive protein, evidence to indicate that any of the other immunoreactive proteins in crude extracts represents an active kinase has yet to be established. The 35kDa protein detected by Western blot that immunoreacts with all three antibodies could likely represent a protein more closely related to authentic $p34^{cdc2}$, but attempts to resolve and correlate this immunoreactive protein with proline directed kinase activity has so far proven unsuccessful.

The recent cloning of the catalytic subunit of the brain PDK conclusively establishes it as a member of the $p34^{cdc2}$ family and substantiates the immunoreactive properties of this enzyme. It is interesting to note from the sequence information that two amino acid changes within the N-terminal region of the brain PDK still enable it to react strongly with N-terminal antibodies whereas three fairly conservative amino acid substitutions within the PSTAIRE region significantly reduce its immunoreactivity towards PSTAIRE antibodies . The N-terminal antibody, although it was made against a peptide sequence that contains certain conserved residues found in all protein kinases, does appear to be specific for cdc2-family members. This antibody along with PSTAIRE and C-terminal peptide antibodies are useful tools for the study of other cdc2-related proteins and can be used for the detection of cdc2-related proteins in other tissues during various purification steps.

The purification of a particular form of a cdc2-related protein enables one to address the possibility that these forms may possess differences in their substrate specificity determinants. In addition, given the immunological and structural relatedness of the brain PDK to $p34^{cdc2}$, the identification of the downstream targets of the brain PDK and their relatedness to known $p34^{cdc2}$ substrates becomes a critical question. The potential substrates for the brain PDK and the localization of likely phosphorylation sites in target proteins is greatly facilitated by a knowledge of the primary determinants or a consensus sequence required for substrate recognition. In the last several years, synthetic peptide substrates have been used to define specific consensus sequence motifs for numerous protein kinases and phosphatases (Kemp and Pearson, 1990, Kennelly and Krebs, 1991). Synthetic peptides, while not a true representation of the phosphorylation sites in native protein substrates, are effective tools for confirming specific determinants. Consensus motifs for protein kinases allow for the identification of additional unknown substrates and provide important information for the design of synthetic substrates that are specific for the enzyme of interest. In addition, information obtained from detailed kinetic analysis of synthetic peptide substrates may prove useful for the design of potential inhibitory peptides.

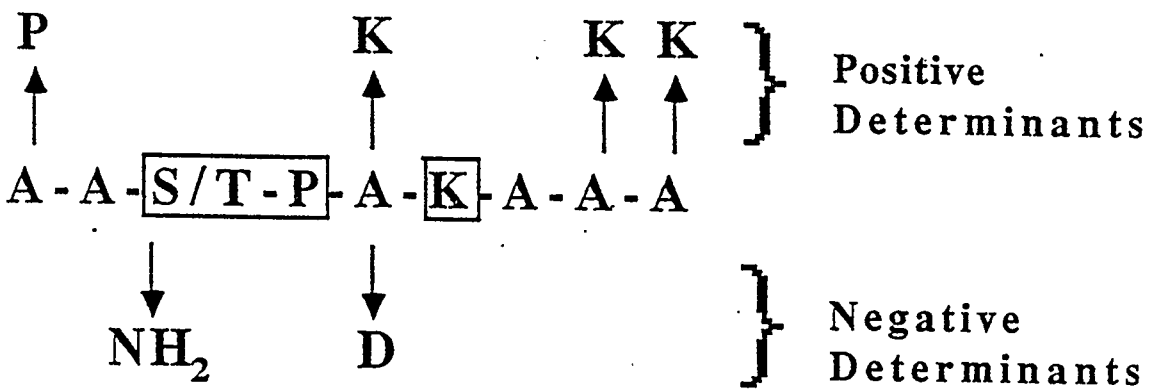
The consensus sequence for $p34^{cdc2}$ recognition was first proposed by Shenoy and coworkers based on identified $pp60^{c\text{-}src}$ phosphorylation sites during mitosis (Shenoy *et al.*, 1989). Moreno and Nurse, when examining the sequence features of six $p34^{cdc2}$ substrate proteins, found that seven out of eight sites conformed to this motif (Moreno and Nurse, 1990). Using kinetic analysis of histone peptide analogues, based on *in vivo* $p34^{cdc2}$ phosphorylation sites, the **S/T**-P-X-K/R motif has essentially been confirmed for the brain PDK, a member of the cdc2-family. The proline residue at the +1 position is indispensable as a substrate determinant, as replacement with an alanine residue abolishes its substrate activity. The basic residue at the +3 position exerts a strong positive influence

on substrate activity, particularly in binding affinity, as replacement with an alanine residue results in a 60-fold increase in K_m value. Finally, serine and threonine residues are preferred phospho-acceptors as a histone peptide having tyrosine substituted for threonine is not a substrate for the brain PDK.

The use of synthetic peptides to elucidate specificity determinants for protein kinases also allows for the investigation of secondary determinants, those which improve substrate activity but are not essential. These are not easily uncovered when phosphorylation sites in native proteins are scanned. In addition, the assessment of kinetics of phosphorylation of specific sites in substrate proteins is usually difficult. In this study, kinetic characterization of synthetic histone peptides has revealed several secondary determinants for the brain PDK (see Figure 13). At the +2 position a basic residue is a positive determinant, while an acidic residue at the same position is a negative determinant for phosphorylation by the brain PDK. Additional basic residues at positions +5 and +6 are positive determinants for phosphorylation of the histone peptide by the brain PDK, while two other secondary determinants, the proline residue at the -2 position and a free α-amino group on the phosphorylatable residue, are found N-terminal to the phosphorylation site. While each of the secondary determinants exerts a relatively small effect on the substrate activity of the histone peptide, their effects appear to be cumulative. For example, substitution of the lysine residue at the +5 or +6 position by alanine each brings about a 5-fold increase in K_m value. If these effects are cumulative, the K_m value for a dual substituted peptide would be expected to increase approximately 25-fold. This is in good agreement with the experimental results comparing K_m values of the dual substituted peptide and the parent peptide HS(9-18) (Table 4). The cumulative effects of secondary determinants on the substrate activity may also to a large extent account for large differences in the K_m values for HS(1-18) when compared to pro-src and bradykinin

Figure 13: Summary of the Positive and Negative Determinants for the Brain PDK

The effects of amino acid substitutions within and surrounding the $p34^{cdc2}$ consensus sequence that alter kinetic parameters for the brain PDK are shown. Amino acid substitutions above the sequence contribute as positive determinants. Amino acid substitutions or the free amino terminus on the phosphorylatable residue below the sequence contribute as negative determinants.


P
K
K K
A-A-S/T-P-A-K-A-A-A
Positive Determinants
NH2
D
Negative Determinants

peptides. All of these peptides contain the consensus motif, yet the K_m values for bradykinin and pro-src are 840 and 340-fold higher respectively than that of HS(1-18). If all of the secondary determinants in HS(9-18) are substituted simultaneously, and the effects of these determinants are cumulative, then the resulting peptide is expected to have K_m values approximately 650 fold higher than the parent peptide. This is comparable to the differences observed for the K_m values for the histone-derived peptide and pro-src and bradykinin.

To a large extent, the substrate structural determinants in addition to those contained in the consensus motif appear to account for the marked differences in K_m values. While most of the determinants in the histone peptide appear to contribute to substrate binding affinity, a comparison of histone peptides, pro-src and bradykinin peptides reveal that there are also differences in relative V_{max}. The determinants accounting for these differences in relative V_{max} have yet to be characterized for the brain PDK and may be attributable to the presence of specific amino acids as yet undefined.

The MAP or ERK kinases are distantly related family members of the cdc2 kinase family in sequence, but are even more diverse with respect to their mechanism of regulation (Pelech and Sanghera, 1992). It is noteworthy that the proline residue at the +1 position is a critical determinant for both the MAP and cdc2 kinases and that the proline residue at the -2 position, also a MAP kinase determinant (Clark-Lewis *et al.*, 1991, Gonzalez *et al.*, 1991), appears to contribute somewhat to efficient substrate phosphorylation by the brain PDK. In a recent review on MAP kinases, Pelech and Sanghera reported unpublished results that efficient phosphorylation of peptide substrates derived from myelin basic protein by $p34^{cdc2}$ also favored the presence of this N-terminal proline, therefore confirming the results of this study (Pelech and Sanghera, 1992). It is unknown if the MAP kinases share additional determinants C-terminal to the phosphorylatable residue that are common to the brain PDK, such as a basic amino acid at the +3 position or the cluster

of basic amino acids further C-terminal to the phosphorylation site. Gonzalez *et al.* in their studies of the phosphorylation of EGF receptor-derived peptides by mammalian MAP kinases, in addition to defining the P-X-**S/T**-P motif, also investigated the role of other secondary determinants (Gonzalez *et al.*, 1991). They found that, outside of this motif, glutamate residues at -1 and +4 positions reduced the rate of phosphorylation by ERK1 and ERK2 kinases compared to peptides having a leucine or alanine residue at these positions. A lysine or arginine residue at the -1 position also contributed as a secondary determinant to favorable phosphorylation of peptide substrates by these two enzymes. This parallels our observations seen with the brain PDK, in that basic residues generally appear to contribute as positive determinants for efficient phosphorylation of substrates and acidic residues contribute as negative determinants. It also lends further support to the overlapping specificities of the cdc2 and MAP kinases. A careful study examining differences in substrate specificity determinants for the MAP kinases and cdc2-family members would address some of these ambiguities.

The phosphorylation sites of native proteins are much more complex than their respective peptide substrates due to higher order structures. This is also likely to apply to substrates of $p34^{cdc2}$ and homologous kinases, although a comparison of the apparent K_m and V_{max} values for native histone H1 and the HS(9-18) peptide indicate that all of the necessary determinants are present in this peptide. Analysis of identified $p34^{cdc2}$ phosphorylation sites in protein substrates, and comparison of these sequences with the $p34^{cdc2}$ consensus motif, reveal that the majority of sites contain the C-terminal proline immediately following the phosphorylatable residue (Table 1). This proline residue, assumed to be essential for $p34^{cdc2}$ and the brain PDK activity, is bypassed in certain identified protein substrates (Chou *et al.*, 1991, Satterwhite *et al.*, 1992). Pines and Hunter have suggested that these sites are phosphorylated possibly because they adopt a similar β-turn conformation to that in which a serine or threonine residue is followed by a proline

(Pines and Hunter, 1990). More exceptions to the motif are noted when examining residues in protein substrates at the +3 position. The requirements for this substrate determinant may be more readily overcome in the higher order structures of protein substrates. The additional secondary determinants elucidated in this study may not be expected to show a very strong constraint on substrate activity of the phosphorylation site in native proteins. Nonetheless, several sites in well established $p34^{cdc2}$ substrates, p53 and $p110^{Rb}$, in addition to histone H1, contain a cluster of basic amino acids C-terminal to the phosphorylation site. Peptides derived from these phosphorylation sites were characterized in a recent study (Marshak *et al.*, 1991) using purified human $p34^{cdc2}$. Both peptides derived from large T-antigen and p53 were reasonable substrates for $p34^{cdc2}$ having K_m values of 74μM and 120μM, respectively. These values are still 10 to 20-fold higher than the reported K_m value for the histone-derived peptide in this study. The peptides derived from p53 and $p110^{Rb}$ differ from the histone-derived peptide with respect to their configuration of basic amino acids relative to the phosphorylation site. Examination of the exact number and spatial requirements of these basic residues that results in the most favored configuration therefore requires further investigation.

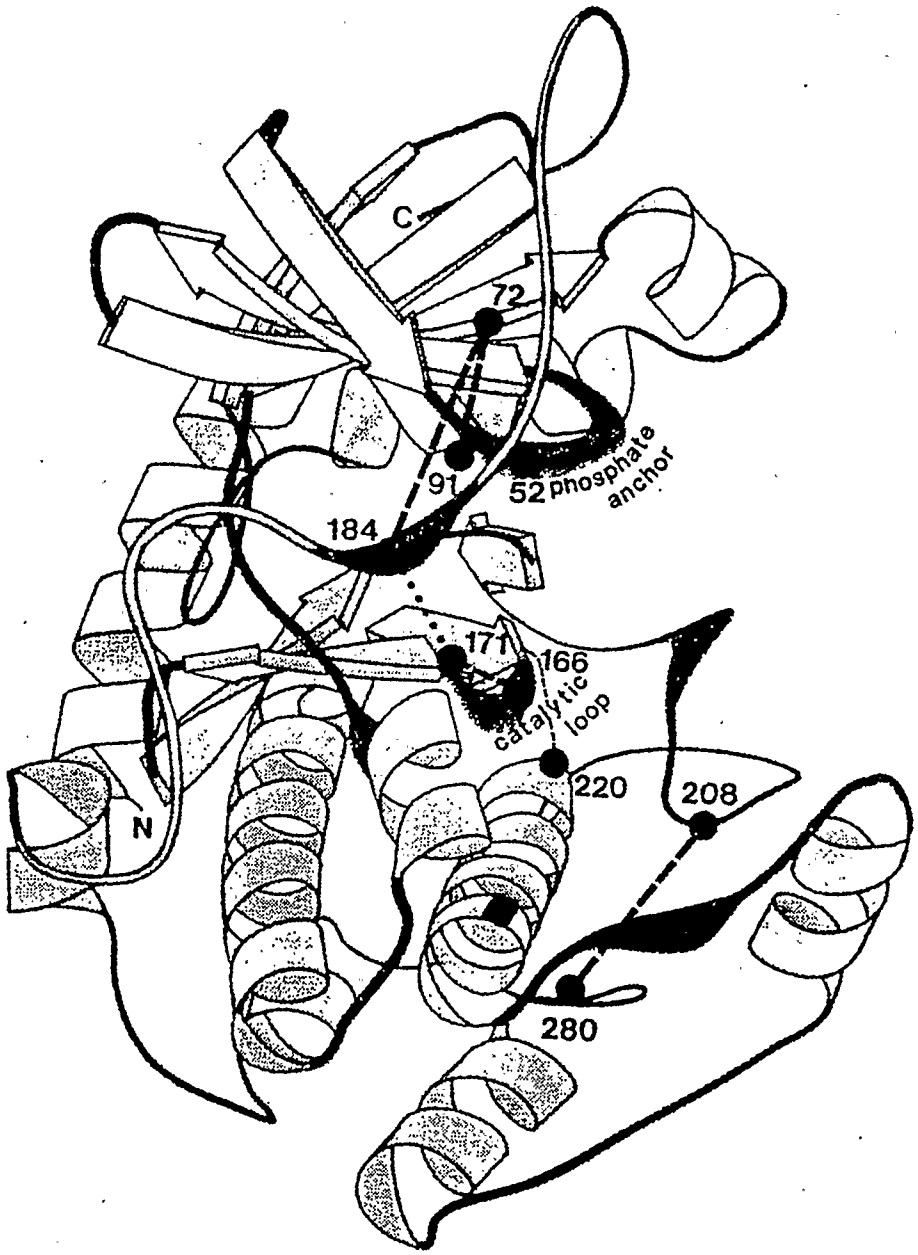
Consensus sequences for the various protein kinases constitute models of the critical substrate recognition determinants for a particular protein kinase. As such, they are expected to be reflected images of their corresponding substrate binding domains within their respective protein kinase (Kennelly and Krebs, 1991). An understanding of how particular regions of a kinase are involved in substrate recognition necessitates a basic knowledge of the higher order structure of protein kinases. In spite of their tremendous diversity, all protein kinases share highly conserved features that, when they are aligned, constitute their catalytic domain (Hanks *et al.*, 1988). This catalytic domain, represented by amino acids 40 to 280 of the cAMP-dependent protein kinase, is subdivided into 11 major conserved subdomains that are separated by regions of more diverse sequence structure

depending on the protein kinase. The elucidation of the crystal structure of the catalytic subunit of the cAMP-dependent protein kinase and its associated inhibitor peptide has provided important clues in understanding how these conserved residues fit into the three dimensional structure of protein kinases and support the very complex nature of kinase/substrate interactions (Knighton *et al.*, 1991a, Knighton *et al.*, 1991b). The fact that all known kinases share this conserved catalytic core allows for the crystal structure of cAMP-dependent protein kinase to serve as a template for viewing the catalytic domains of other protein kinases. Briefly, the crystal structure of the A-kinase catalytic subunit, represented by a ribbon diagram in Figure 14, consists of a bi-lobed structure, separated by a cleft between the two lobes. The N-terminal portion of the protein, from amino acid residues 40 to 125, is associated with the small lobe. It contains predominantly β-strands, but has one α-helical region. Early chemical data along with the crystal structure have verified the function of this region to be associated with nucleotide binding (Knighton *et al.*, 1991a, Knighton *et al.*, 1991b). The C-terminal region of the protein constitutes the core of the large lobe which is predominantly helical in structure. The large lobe of A-kinase contains nearly all of the features important for peptide recognition, as the consensus region for the A-kinase inhibitor peptide lies along the surface of the cleft corresponding to the large lobe (Knighton *et al.*, 1991a).

Given the favorable properties of basic residues within the histone-derived peptide substrate on both $p34^{cdc2}$ and the brain PDK activity, a corresponding acidic domain in these kinases may function as a component of the substrate binding domain. There is a cluster of acidic amino acids just preceding the PSTAIRE region of human $p34^{cdc2}$ and several of its homologues including the brain PDK. This region has been shown by site directed mutagenesis of *S. pombe* $p34^{cdc2}$ to be important for cyclin binding (Brambilla *et al.*, 1991). Alignment of the $p34^{cdc2}$ sequence with the A-kinase crystal structure reveals that this acidic cluster (corresponding to amino acids 77 to 81 of the A-kinase sequence) is

Figure 14: Schematic Diagram of the Crystal Structure of the cAMP-dependent Protein Kinase

A ribbon diagram representing the crystal structure of the cAMP-dependent protein kinase is shown (Knighton *et al.*, 1991a).


C
72
91
52
Phosphate anchor
184
171
166
catalytic loop
220
208
N
280

predicted to be located in a single α-helical region of the small lobe that lies above the cleft. It can be speculated that this region may function as part of the substrate binding domain, forming some sort of electrostatic interaction with the basic residues of the histone-substrate peptides. The identification of structural determinants involved in binding the A-kinase inhibitor peptide to be localized in the large lobe and evidence to suggest the involvement of this basic region in cyclin binding, do not support this notion. In addition the bend or kink that the consensus proline is predicted to exert in substrate proteins might alter the structure such that the basic residues are directed away from this acidic cluster when the substrate is bound to the enzyme. Nonetheless, if the acidic cluster does not function as part of the substrate binding domain, it may well function as a targeting domain, with its corresponding bound cyclin, directing substrate proteins to the enzyme active site. Preliminary studies have also indicated that $p34^{cdc2}$ activity is inhibited by polylysine. The acidic box in $p34^{cdc2}$ also makes this an attractive site for interaction with polylysine. Given the involvement of this region in cyclin binding, polylysine could effectively be inhibiting $p34^{cdc2}$ kinase activity through displacement of its cyclin regulatory subunit. The answers to some of these questions could be addressed using site directed mutagenesis of these acidic residues in $p34^{cdc2}$ or the brain PDK and examining the kinetic properties of these mutated forms towards various histone-derived peptides and the effects of polycations on their activity. Ultimately, resolution of the crystal structure of $p34^{cdc2}$ complexed with cyclin and its substrate peptide would lend further insight into its mechanism of catalysis and address the role of the acidic cluster in $p34^{cdc2}$ homologues and its potential involvement in substrate binding and/or binding of polycation inhibitors.

Several interesting analogies can be drawn from the identified structural determinants for the brain PDK deduced in this study and what is known about the structural determinants for another protein kinase, casein kinase II. Casein kinase II, a widely distributed tetrameric serine/threonine kinase composed of two α subunits and two

β subunits, is likely to play a role in a wide variety of signal transduction events (Pinna, 1990 for review). As basic residues within histone-derived peptides are favorable determinants for the brain PDK and $p34^{cdc2}$, substrate specificity determinants for casein kinase II have been shown to require the presence of acidic amino acid residues immediately C-terminal to the phosphorylation site from positions +1 to +3, with the optimum position being +3 (Kemp, 1990, Kennelly and Krebs, 1991). Optimizing factors for the efficient phosphorylation of substrate peptides, in addition to the acidic residues at the +3 position, include the presence of acidic residues N-terminal to the phosphorylation site or an extension of acidic amino acids further C-terminal to the +3 position (Kemp, 1990). The overall number of acidic residues in substrate peptides is critical for determining substrate binding affinity by lowering K_m values. Whereas phosphorylation of histone-derived peptides is hampered by the presence of acidic amino acids within the consensus sequence, casein kinase II catalyzed phosphorylation is hampered by the presence of basic amino acids both C-terminal and N-terminal to the phosphorylation site of substrate peptides, in positions where neutral amino acid substitutions on substrate peptides do not affect kinetic parameters. Casein kinase II is also inhibited by the polyanions heparin and polyaspartate and stimulated by polycations (Pinna, 1990). Alignment of the amino acid sequence of casein kinase II with that of A-kinase also reveals a polycation stretch of amino acids highly conserved among casein kinase II family members in the same region as the acidic cluster for $p34^{cdc2}$ and the brain PDK (Hanks *et al.*, 1988). It has been suggested that this region in casein kinase II could facilitate substrate binding since preferred casein kinase II targets contain acidic residues (Pinna, 1990). Site directed mutagenesis of the casein kinase II α subunit, mutating two lysine residues within this polycation stretch, increased the IC_{50} for heparin inhibition 70-fold, but had no effect on the binding affinity of substrates (Hu and Rubin, 1990). This implied that this basic stretch in casein kinase II was important for heparin binding but not for substrate binding. This was further

supported by studies showing that peptides made against this basic cluster of amino acids could effectively reverse heparin inhibition of casein kinase II (Charlton *et al.*, 1992).

In spite of the very intensive studies of cdc2 kinase in the last several years, this work represents the first attempt to investigate the substrate determinants for a purified cdc2-family member using synthetic peptides. We have used synthetic peptide substrates derived from histone H1 to characterize the determinants for a novel cdc2-like kinase purified from bovine brain. The data presented in this study demonstrate that both the brain PDK and $p34^{cdc2}$ share common *in vitro* specificity determinants, although a detailed analysis of the substrate specificity for $p34^{cdc2}$ and its various forms has not yet been undertaken. While this present study has defined the structural determinants of substrates for the brain PDK, it can also provide us with some insight into $p34^{cdc2}$ kinase substrate specificity. With the existence of multiple forms of cdc2 kinases and cyclins, and evidence to suggest that different forms have different *in vitro* substrate specificities (Brizuela *et al.*, 1989, Elledge *et al.*, 1992, Matsushime *et al.*, 1992), the set of substrate peptides prepared in this study may be useful in defining possible differences in substrate specificity for these various forms. Such studies await the availability of purified samples of the various cdc2 family members and their respective complexes.

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