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Substrate specificity of wheat embryo calcium-dependent protein kinase

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Wheat embryo Ca^{2+}-dependent protein kinase (CDPK) phosphorylates a variety of synthetic peptides having a Basic-X-X-Ser sequence but peptides with a Basic-Basic-X-Ser(Thr) sequence are relatively poor substrates. Wheat germ CDPK phosphorylates a variety of proteins of which histone H1 and bovine serum albumin are among the better substrates. A single phosphorylated tryptic peptide was purified from bovine histone H1 phosphorylated by wheat embryo CDPK and subjected to Edman degradation yielding the sequence Gly^{97}-Thr-Gly-Ala-Ser-Gly-Ser(PO_4)-Phe-Lys^{105}. Ser^{103} on bovine histone H1 is also the residue phosphorylated by rat brain protein kinase C.

Ca^{2+}; Protein kinase; Histone H1

1. INTRODUCTION

Ca^{2+}-dependent protein kinases (CDPKs) have been resolved from higher plants [1–5] and are likely to be involved in Ca^{2+}-mediated signal transduction in plants [1] as are such enzymes in animal systems [6,7]. Plant CDPKs have some properties in common with animal protein kinase C [1–4]. However, little is known of the substrate specificity of plant CDPKs. The present paper describes the amino acid sequence specificity of wheat embryo CDPK.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (3 Ci/mmol) was obtained from Amersham International, UK. $S6_{229-239}$ and related peptides were synthesized by Dr B.E. Kemp [8]. Other synthetic peptides were obtained from Auspep, Melbourne, Australia. Histone H1 was obtained from Boehringer. Calf thymus histones (types II-AS, III-S, V-S, VI-S, VII-S and VIII-S) and all other proteins were obtained from the Sigma Chemical Co. Wheat embryo CDPK was purified as described previously [3]. TPCK-treated trypsin was obtained from Worthington Biochemical Corp.

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2.2. *Protein phosphorylation*

Protein phosphorylation was routinely measured radiochemically by precipitation on phosphocellulose (P81) disks as previously described [2] in a standard reaction medium containing 62.5 mM Tris (Cl^-, pH 8.0), 8 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 mM 2-mercaptoethanol, 0.25 mM EGTA, 1.0 mM $CaCl_2$, 25 μM ATP (spec. act. of [γ-^{32}P]ATP was ~100 mCi/mmol), 0.5 mg/ml protein substrate and wheat embryo CDPK. Phosphoamino acid analysis of phosphorylated polypeptides by acid hydrolysis and high-voltage electrophoresis and SDS-PAGE of phosphorylated proteins and autoradiography were conducted as described previously [5]. K_m values were determined by fitting kinetic data to the Michaelis-Menten equation by a least squares curve-fitting program.

2.3. *Histone H1 phosphorylation site sequence*

250 μg ^{32}P-bovine histone H1 (phosphorylated to 0.5 mol/mol by wheat germ CDPK) was precipitated by cold 17% (w/v) trichloroacetic acid, washed with ethanol/ether (1:1, v/v) and dissolved in 50 mM Tris (Cl^-, pH 8.0)/2 mM 2-mercaptoethanol. After tryptic digestion (trypsin/histone H1 1:30 (w/w) for 22 h at 20°C), proteolysis was terminated by addition of 0.5% (v/v) trifluoroacetic acid. 125 μg trypsin-treated [^{32}P]histone H1 was subjected to reversed-phase HPLC on a

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C_{18} column (4.6 mm × 25 cm; 5 μ particle size; Altech Associates) with an Aquapore RP-300 guard column (4.6 mm×30 mm; 7 μ particle size; Brownlee Laboratories) using an acetonitrile gradient in the presence of aqueous 0.1% (v/v) trifluoroacetic acid (linear gradient increasing from 0 to 50% acetonitrile in 100 min; flow rate 1 ml·min^{-1}). A single [^{32}P]peptide was obtained and was subjected to Edman degradation employing an Applied Biosystems 470A gas phase peptide sequenator. PTH amino acid analysis employed a Waters HPLC fitted with a Zorbax C8 column (Du Pont, Wilmington, DA) which was eluted with a discontinuous sodium acetate buffer (pH 5.0)/acetonitrile gradient [9].

3. RESULTS AND DISCUSSION

Wheat embryo Ca^{2+}-dependent protein kinase (CDPK) from wheat germ catalyzes the phosphorylation of a variety of proteins of which the best substrates are heterogeneous protein preparations including dephosphorylated casein and histone preparations III-S, V-S and VII-S and purified preparations of histone H1, bovine serum albumin and rabbit muscle protein kinase inhibitor protein (table 1). A variety of other proteins are much poorer substrates for wheat embryo CDPK, namely heterogeneous preparations including myosin light chains, II-AS, VI-S and VIII-S histone preparations and homogeneous preparations of actin, histone H2B, human serum albumin and phosvitin (table 1). Electrophoresis of phosphorylated proteins and autoradiography of the dried gels identified the major polypeptides phosphorylated in the protein preparations used (table 1). Ovalbumin, phosphorylase *b*, histone H4, protamine and rabbit liver metallothioneins I and II are not phosphorylated by wheat embryo CDPK. The K_m values for the better substrates in the standard conditions are as follows (K_m values in parentheses): histone H1 (5.9 ± 1.8 μM), bovine serum albumin (31 ± 13 μM), III-S (0.6 ± 0.3 mg·ml^{-1}) and VII-S (0.2 ± 0.1 mg·ml^{-1}).

A variety of synthetic peptides are phosphorylated by wheat embryo CDPK (table 2). The significantly phosphorylated peptides (2–9) all contain a RXXS sequence. Phosphoamino acid analysis, as described in section 2, revealed only [^{32}P]phosphoserine (and no [^{32}P]phosphothreonine) from ^{32}P-peptides 1–4 of table 2. Thus peptides 3 and 4 are unambiguously labelled on serines 7 and 9, respectively. Serine phosphorylations are therefore likely on the homologous serine 7 (peptide 2), serine 9 (peptide 5) and serine

Table 1

Substrate specificity of wheat embryo CDPK

Substrate	Phosphorylation rate[a] (% control)	Phosphopeptide apparent M_r ($\times 10^{-3}$)[b]
III-S histones	100	32,29,24,22, 20,19,17
V-S histones	36	34,31,24,17
Histone H1	35	31
Bovine serum albumin	25	67
Casein	10	67,29,24,8
VII-S histones	10	17
Protein kinase inhibitor (rabbit)	5	16
VI-S histones	2	17,15,13
Actin	2	55
Histone H2B	2	17
Human serum albumin	2	67
Myosin light chains (rabbit)	1	24,20
II-AS histones	1	17,14
Phosvitin	1	24
VIII-S histones	0.7	17,15
PEP carboxylase	0.4	–
Myosin light chains (chicken)	0.3	19,16

[a] Protein phosphorylation rates with 0.5 mg/ml protein substrate were determined as described in section 2 and are expressed as % of the rate with histone preparation III-S as substrate (100%)

[b] The apparent M_r values for the major ^{32}P-labelled peptides were determined by SDS-PAGE and autoradiography

Table 2

Phosphorylation of synthetic peptides by wheat embryo CDPK

Substrate[a]	Phosphorylation rate[b] (% control)
(1) III-S	100
(2) PLSRTLSVAAKK-NH_2	458
(3) PLRRTLSVAA-NH_2	256
(4) KKRAARATSNVFA-NH_2	116
(5) AKRPQRATSNVFS-NH_2	45
(6) AKRRRLASLRA-NH_2	9
(7) AKRRRLSSLRA-NH_2	8
(8) AKRRRLSALRA-NH_2	7
(9) AKRRRLSSLAA-NH_2	6
(10) LRRASLG	0.5
(11) RKRSRKE	0
(12) VRKRTLRRL-NH_2	0
(13) KRRISGL-NH_2	0

[a] Synthetic peptide substrates were included at 0.25 mg/ml

[b] Phosphorylation rate is expressed as % of control rate with 0.5 mg/ml III-S as substrate

8 (peptide 7) which share the RXXS sequence (table 2). Phosphoamino acid analysis of phosphorylated peptide 5 revealed both serine and threonine phosphorylation ([^{32}P]Ser(PO_4):[^{32}P]Thr(PO_4) approx. 0.8). Peptides 10–13, which did not have a RXXS sequence, are not significantly phosphorylated by wheat embryo CDPK. The two best substrates (peptides 2 and 3) have a RTXS sequence as compared to a RXTS sequence of the next best substrates (peptides 4 and 5) (table 2). Peptide 7 ($S6_{229-239}$) corresponds to a major phosphorylatable sequence of mammalian ribosomal protein S6 [8]. The K_m for peptide 7 is 23 ± 8 μM as compared to 0.5 μM for rat brain protein kinase C [8]. The A-235 or A-236 substitutions to peptide 7 (peptides 6 and 8, respectively) do not markedly affect phosphorylation rate (table 2), indicating that either RXS or RXXS can serve as the recognition sequence. The lack of substantial effect of the A-238 substitution (peptide 9) indicates that, unlike the situation with protein kinase C [8], the C-terminal side R does not affect peptide 7 phosphorylation (table 2).

Purified calf thymus histone H1 was phosphorylated using [γ-^{32}P]ATP in a CDPK-catalyzed reaction (phosphorylation stoichiometry, 0.5 mol/mol histone H1) and subjected to tryptic digestion and reversed phase HPLC analysis of the tryptic peptides as described in section 2. Only one ^{32}P-labelled tryptic peptide peak was recovered which accounted for 64% of the radiolabel present in the undigested protein. Sequencing of this peptide revealed an unambiguous amino acid sequence which corresponded to the tryptic phosphopeptide derived from phosphorylation of bovine histone H1 by protein kinase C [10], namely $G^{97}TGASGS(PO_4)FK^{105}$. The assignment of the phosphorylation site is based on the absence of the PTH-serine in the 7th cycle of the Edman degradation sequence corresponding to Ser^{103} of calf thymus histone H1, PTH-phosphoserine being unstable in the Edman cycle conditions [11]. The $S^{101}GS$ sequence [10] was confirmed by the elevation of serine-derived PTH-dehydroalanine and its dithiothreitol adduct (see [12]) in cycles 5 and 7, respectively (fig.1).

The histone H1 sequence phosphorylated with high stoichiometry (0.5 mol/mol) by wheat germ CDPK is identical to that phosphorylated by animal protein kinase C [10], but differs in general

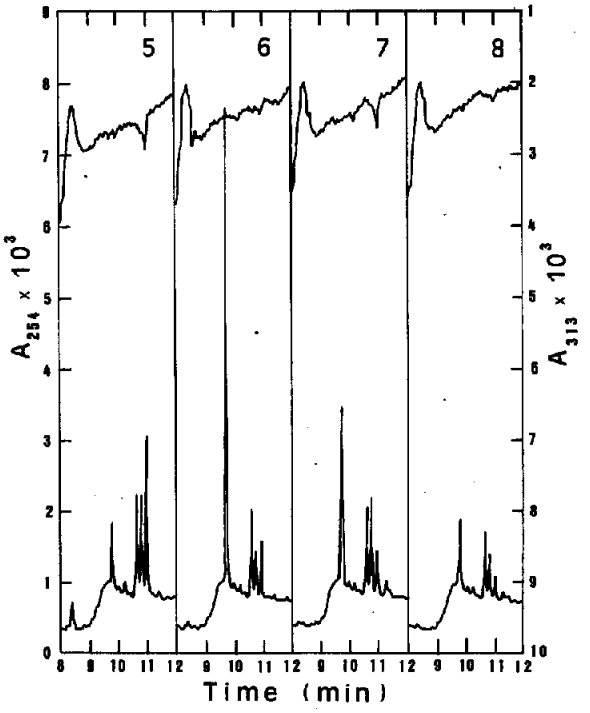

Fig.1. Cycles 5, 6, 7 and 8 of the Edman degradation of the ^{32}P-labelled tryptic peptide from [^{32}P]histone H1. PTH-serine and PTH-dehydroalanine [19] elute at retention times of 8.4 and 10.8 min, respectively (A_{254} profile); the dithiothreitol adduct of PTH-dehydroalanine [19] elutes at 11.0 min (A_{313} profile). Cycles 5, 6, 7 and 8 correspond to S^{101}, G^{102}, S^{103} and F^{104}. PTH-glycine elutes at 9.8 min (cycle 6); PTH-phenylalanine elutes at 15.4 min (cycle 8; not shown).

structure from the Basic-Basic-X-Ser sequence phosphorylated by cAMP-dependent protein kinase in histone H1 and other proteins [13]. Consistent with this difference, Kemptide (peptide 10), a good cAMP-dependent protein kinase substrate [14], is a very poor substrate for the wheat germ CDPK as are other peptides containing a Basic-Basic-X-Ser(or Thr) sequence (peptides 11, 12 and 13) (table 2). Peptide 11 is a good substrate of cGMP-dependent protein kinase [15].

Peptides 2 and 3 (analogues derived from glycogen synthase) [8] and peptide 7 (all of which contain a Basic-X-X-Ser sequence) are all phosphorylated by wheat germ CDPK (table 2), cyclic AMP-dependent protein kinase and protein kinase C [8] and by multifunctional calmodulin-dependent protein kinase [16]. However wheat germ CDPK differs from multifunctional

calmodulin-dependent protein kinase in that histone H1 is a very poor substrate for the latter enzyme [17]. Wheat germ CDPK also differs from protein kinase C in that it does not phosphorylate the EGF receptor (650–658) peptide analogue (peptide 12), which is an excellent substrate for protein kinase C [8]. Peptides 4 and 5 (synthetic analogues of the myosin light chain phosphorylation region) [18] are phosphorylated by wheat germ CDPK (table 2). However myosin light chains are very poor substrates for wheat embryo CDPK (table 1) while, conversely, casein and histones are relatively poor substrates for myosin light chain kinases [18] (cf. table 2).

The H1 sequence N-terminal to the Ser^{103} phosphorylated by wheat germ CDPK and protein kinase C departs from the Basic-X-X-Ser mode recognized by these enzymes (table 2) [8]. While Ser^{103} is well removed from any N-terminal Basic-Basic sequence (the nearest being Lys-Lys^{63}) [19], there is a basic residue (Lys^{105}) on the COOH-terminal side. It therefore appears that, as shown for protein kinase C [8,10,20], basic residues on either side of the phosphorylation site may influence the site specificity of wheat germ CDPK.

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