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Phosphorylation of phosvitin by casein kinase-2 provides the evidence that phosphoserines can replace carboxylic amino acids as specificity determinants

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The consensus sequence of casein kinase-2 consists of a serine (threonine) followed by a cluster of glutamic and/or aspartic acids, the one at position +3 playing an especially crucial role (Marin et al., (1986) Eur. J. Biochem. 160, 239–244 and Kuenzel et al. (1987) J. Biol. Chem. 262, 9136–9140). None of the 123 serines of the main phosvitin component (34 kDa) fulfils such a requirement (Byrne et al. (1984) Biochemistry 23, 4275–4279), rather, most of them are clustered into stretches of up to 14 entirely phosphorylated residues. Three out of the four threonines lie close to the N-terminal side of such phosphoseryl blocks. Here we show that native 34 kDa phosvitin is a poor substrate of casein kinase-2, its radiolabeling occurring mostly at threonine residue(s); a very slight (1%) previous dephosphorylation with acid phosphatase converts phosvitin into an excellent substrate for casein kinase-2, its phosphorylation occurring almost exclusively at serine residues. Extensive dephosphorylation however (greater than 40%) reduces the phosphorylation efficiency of casein kinase-2. These results show that phosphoserine residues can replace carboxylic residues as specificity determinants for casein kinase-2.

Casein kinases are multifunctional enzymes involved in the regulation of many biological processes, operationally termed after their preference for casein over histones as in vitro phosphorylatable substrates (reviewed in Refs. 1 and 2). They are independent of cyclic nucleotides and calcium and can be grouped into two families casein kinase 1 and casein kinase-2 – according to the different structure, specificity and response to heparin and polycations. Although nowaday casein is most commonly used for assaying casein kinases in vitro, phosvitin is also an excellent substrate and was largely employed in the early studies on these enzymes (e.g., Refs. 3–6), consequently, also termed 'phosvitin kinase'. Its unusual amino acid composition, including over 55% almost entirely phosphorylated serines [7], until recently has hampered the elucidation of the primary structure of phosvitin which could not be exploited for assessing the structural requirements of casein kinases.

An exception was provided by a pioneer study of 1975 in which we reported that by far most of the ^{32}P incorporated into phosvitin by a casein kinase later identified as casein kinase-2, was recovered in extremely acidic fragments composed of several phosphorylated residues virtually devoid of any other amino acids [8]. Considering that over 95% of the hydroxylic residues of phosvitin

Abbreviation: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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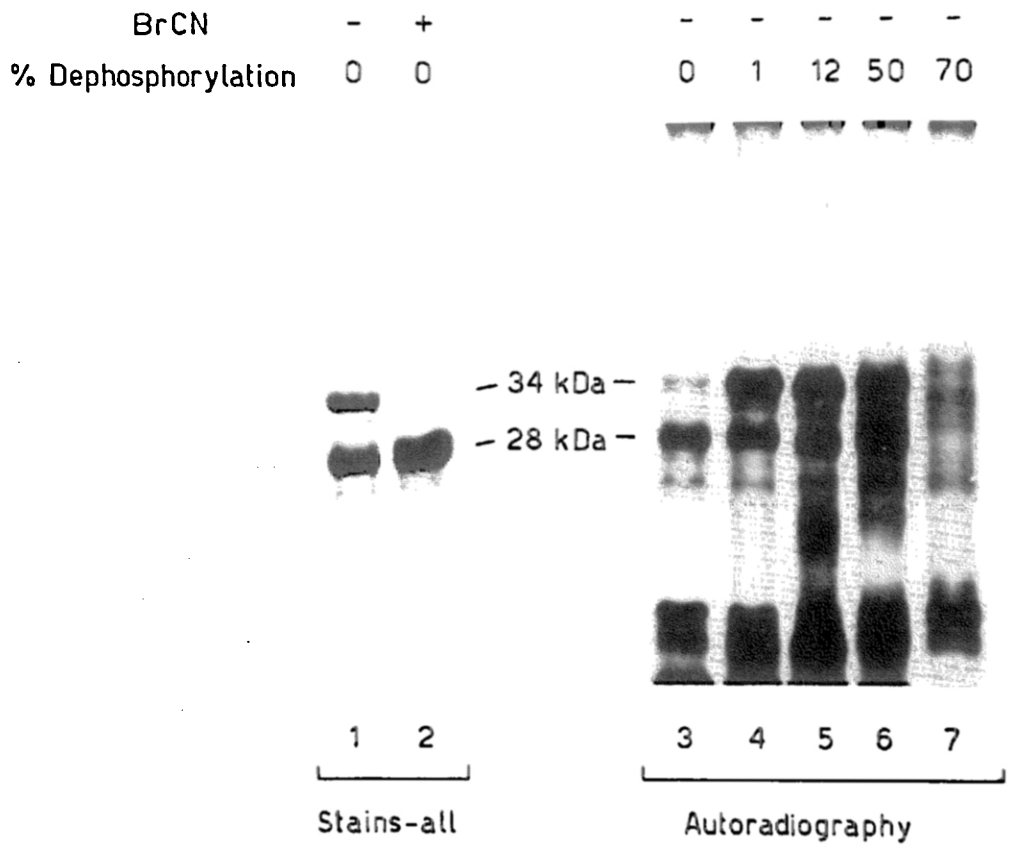

Fig. 1. Phosphorylation of the 34 kDa component of phosvitin by casein kinase-2: effect of previous partial dephosphorylation. Phosvitin (from Sigma) was partially dephosphorylated by incubating 20 mg aliquots for various periods of time with potato acid phosphatase [15]. The reaction was stopped by boiling for 5 min, and the inorganic phosphate released was evaluated [16]. Under our conditions approx. 1% dephosphorylation was achieved after 7 min incubation. The samples were dialyzed against 5 mM Tris-HCl buffer (pH 7.5). Phosphorylation was performed and evaluated essentially as described in Ref. 17 by incubation of either native or variably dephosphorylated phosvitins (final concentration 2 mg/ml) with purified casein kinase-2 from rat liver cytosol, followed by SDS-PAGE. Phosvitin bands were evidenced by stains-all treatment [14] and their radiolabelling was evaluated by autoradiography of the dried gel (lanes 3–7). Lanes 1 and 2 refer to the stains-all patterns of phosvitin and CNBr-digested phosvitin, respectively. The molecular masses of the two main phosvitin bands (34 and 28 kDa) were established by calibration with LKB molecular weight markers (lane not shown). Upon CNBr cleavage at Met-39 the 34 kDa component gives rise to 29 kDa product, slightly more retarded than the 28 kDa band.

are already phosphorylated in the native molecule, this would mean that the residues newly phosphorylated by casein kinase-2 are either within or adjacent to pre-existing phosphoserine residues.

Some recent findings elicited new interest in that old observation. (1) Both the inspection of the sites phosphorylated by casein kinase-2 in its physiological tragets and studies with synthetic peptides have unambiguously shown that the specificity determinant of casein kinase-2 consist of an acidic cluster composed of either glutamic acid [9,10] or aspartic acid [11] on the C terminal side of the target residue, the carboxylic residue at position +3 playing an especially crucial role [10]. (2) The elucidation of the primary structure of hen phosvitin [12] revealed that none of its 123 serines exhibits such a structural feature. Assuming, therefore, that only glutamic and aspartic acids can act as specificity determinants for casein kinase-2, phosvitin should not be a substrate for this kinase. Most of phosvitin serines however are clustered into long stretches of up to 14 entirely

phosphorylated consecutive residues. (3) Three out of the four threonine residues which are largely unphosphorylated in native phosvitin [7] are also located on the N-terminal side of stretches of consecutive phosphoserine residues [12].

Altogether these observations strongly suggest that if in fact phosvitin, rather than contaminant proteins, is affected by casein kinase-2, phosphorylated side chains should be able to replace carboxylic amino acids as specificity determinants. In this paper, we fully convalidate such a concept by showing that:

(1) Phosvitin is indeed phosphorylated by casein kinase-2. Such a control was necessary because phosvitin is not homogenous [13], being composed of a more abundant 34 kDa polypeptide including a single methionine, whose primary structure has since been elucidated [12], and by a 28 kDa component lacking methionine whose amino acid sequence is still unknown. Low molecular mass contaminants are also present in most phosvitin preparations. As shown in Fig. 1, the 34 kDa phosvitin band is significantly phosphorylated by casein kinase-2, although other components are more intensely radiolabeled. The radioactivity incorporated into the 34 kDa band is mostly accounted for by Thr-^{32}P (Table I). The identification of the 34 kDa band with authentic phosvitin, based on its electrophoretic mobility and blue color with stains-all [14] was further confirmed by its susceptibility to CNBr cleavage (see Fig. 1, lane 2), at its unique Met-39, content whereas the smaller 28 kDa component is devoid of methionine and consequently insensitive to CNBr treatment [18].

(2) A previous very slight dephosphorylation of phosvitin (1%) with acid phosphatase results in a dramatic and specific increase of the 34 kDa band phosphorylation by casein kinase-2 without affecting the phosphorylation of the 28 kDa band (Fig. 1, lane 4). However, while threonine is predominantly phosphorylated in native 34 kDa phosvitin, Ser-^{32}P mainly accounts for the radioactivity incorporated into slightly dephosphorylated phosvitin (Table I). Fig. 1 also shows that more pronounced previous dephosphorylation promotes a decrease of phosphorylation rate by casein kinase-2.

(3) The discovery made previously [8] that most of the radioactivity incorporated by casein kinase-2 is associated with the polyphosphorylated fragments isolated after partial acid hydrolysis ((Ser-P)$_n$ clusters), has been confirmed for the individual 34 kDa band of authentic phosvitin: both Thr-^{32}P and Ser-^{32}P incorporated into the 34 kDa component of 1% dephosphorylated phosvitin were recovered in anodic peptides running ahead of P_i and, thus, exhibiting the typical mobility of multiphosphorylated clusters (Fig. 2). The failure to detect appreciable amounts of free Ser-^{32}P in 12 M HCl hydrolysates is consistent with the reported stability of (Ser-P)$_n$ clusters under these conditions [20] and supports the view that isolated serines not included into phosphoserine stretches are unaffected by casein kinase-2.

All the above data are consistent with the assumption that (Ser-P)$_n$ stretches present in phosvitin molecule act as specificity determinants for the phosphorylation by casein kinase-2 instead of the clusters of glutamic and/or aspartic acids located on the C-terminal side of the residues phosphorylated by this protein kinase in the other known substrates. Three out of the four threonines potentially accounting for the phosphorylation of native phosvitin by casein kinase-2 are indeed located close to the N-terminal end of rows of six consecutive phosphoserines [12] and are only partially occupied by unlabelled phosphate [13]. On the other hand, upon slight dephosphorylation, a

TABLE I

SERINE AND THREONINE PHOSPHORYLATION IN THE 34 KDA COMPONENT OF NATIVE AND PARTIALLY DEPHOSPHORYLATED PHOSVITIN INCUBATED WITH CASEIN KINASE-2

The radiolabeled 34 kDa bands isolated as in Fig. 1 (lanes 3–5) were excised and swollen overnight by continuous stirring in 50 mM Tris-HCl (pH 7.5) containing 1% SDS. The eluted protein was precipitated with ice-cold acetone and the pellet was analyzed for its Ser-^{32}P and Thr-^{32}P content by partial acid hydrolysis followed by pH 1.9 high-voltage paper electrophoresis, as described in Ref. 19.

Phosvitin	^{32}P incorporated (CPM)		Ratio Ser-^{32}P / Thr-^{32}P
	Ser-^{32}P	Thr-^{32}P	
Native	1313	2081	0.63
1% dephosphorylated	17162	4160	4.12
12% dephosphorylated	15720	3785	4.15

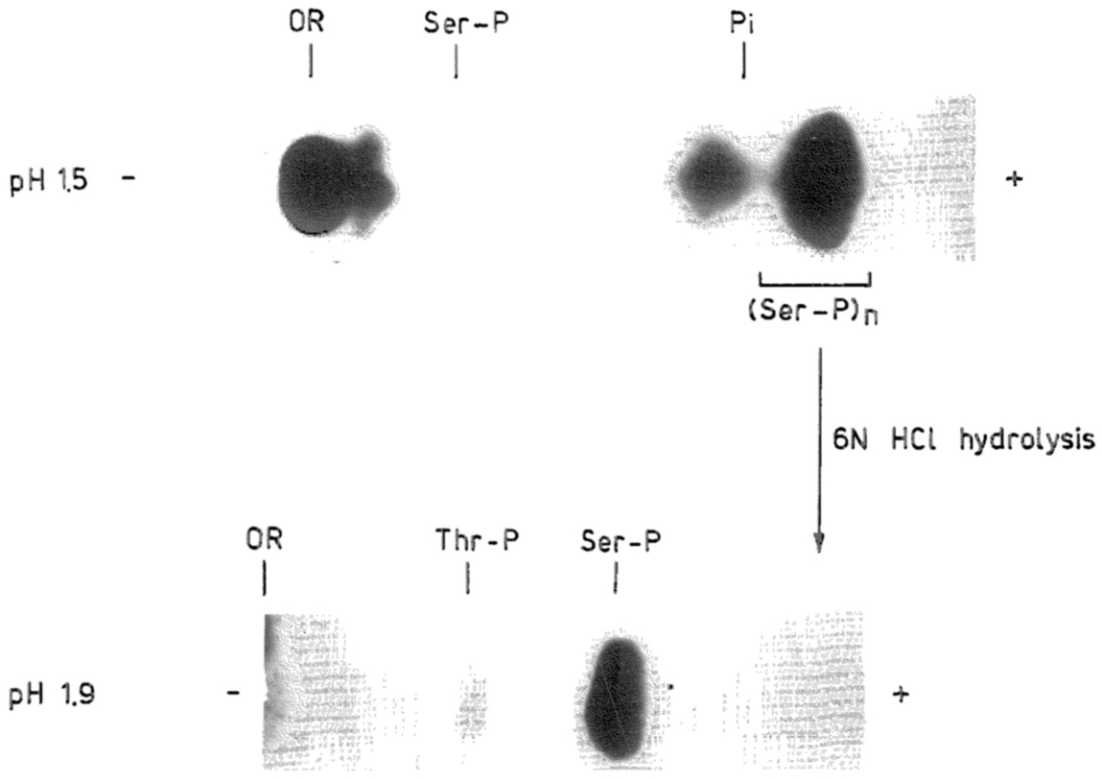

Fig. 2. Isolation of radiolabeled clusters of consecutive phosphorylated residues from 34 kDa phosvitin phosphorylated by casein kinase-2. 1% dephosphorylated phosvitin was phosphorylated by incubation with casein kinase-2 and subjected to SDS-PAGE as described in Fig. 1. The radiolabeled 34 kDa band, eluted as in Table I was subjected to 12 M HCl hydrolysis at 37 °C for 18 h and $(Ser\text{-}P)_n$ clusters were isolated by pH 1.5 high-voltage paper electrophoresis, essentially according to Williams and Sanger [20]. The corresponding autoradiography is shown in the upper part of the figure. The anodic radioactive spot running ahead of inorganic phosphate, with the typical mobility of $(Ser\text{-}P)_n$ ($n > 2$) peptides [8,20,21] was eluted with distilled water, subjected to 6 M HCl hydrolysis and analyzed for its phosphoaminoacid content by pH 1.9 high-voltage paper electrophoresis as described in Ref. 19. The autoradiography is shown.

few unoccupied serines are randomly made available, some of which are presumably located in the initial part of the phosphoserine clusters which are still mostly phosphorylated. Conceivably such residues are responsible for the overwhelming radiolabeling of serine over threonine in partially dephosphorylated phosvitin (Table I and Fig. 2), consistent with the preference for serine over threonine of casein kinase-2, under comparable conditions [10,19]. As expected, more extensive dephosphorylation hinders both serine and threonine phosphorylation by gradually suppressing the specificity determinants.

Quite similar results have been obtained recently using the synthetic phosphopeptide AcSer(P)-Ser(P)-Ser(P): phosphorylation by casein kinase-2 requires a previous partial dephosphorylation while more extensive dephosphorylation (greater than 30%) slows down the casein kinase reaction (unpublished data in collaboration with Perich, J.W. and Johns, R.B.).

The demonstration, provided by phosvitin phosphorylation, of specificity of casein kinase-2 be determined by phosphoserines, besides glutamic and aspartic acids, could be of physiological relevance, disclosing the possibility that previous phosphorylation by another protein kinase may trigger the subsequent phosphorylation by casein kinase-2, which in turn has been shown to potentiate the subsequent phosphorylation of some protein substrates by glycogen synthase kinase-3 (F_A kinase) [22–24]. It will be interesting, in this re-

spect, to determine whether phosphotyrosine, besides phosphoserine, could serve as a specificity determinant for casein kinase-2, thus providing a potential mechanism by which a given serine/threonine-protein kinase might undergo substrate directed regulation by a tyrosine-specific one.

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