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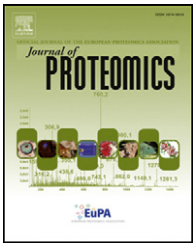

Topographical distribution of phosphorylation sites of phosvitins by mass spectrometry

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ABSTRACT

Phosvitin, derived from the vitellogenin II gene protein, is a highly phosphorylated protein found in egg yolk. A second hypothetical protein has been predicted based on the vitellogenin I gene, but has not been defined at the protein level. Mass spectrometric analysis was used to identify the phosphopeptide sequences and the precise sites of phosphorylation of two phosvitins, phosvitin 1 and phosvitin 2 derived from vitellogenins I and II, respectively. Samples of native phosvitin were subjected to tryptic digestion followed by mass spectrometric analysis: (i) native phosvitin peptides, (ii) after treatment with NaOH, and (iii) after chemical derivatization of P-Ser/P-Thr residues by dithiothreitol under base-catalyzed conditions. A combination of these approaches led to the identification of 68 and 35 phosphopeptides with 89 (81 P-Ser and 8 P-Thr residues) and 62 (57 P-Ser and 5 P-Thr residues) phosphorylation sites of phosvitin 1 and phosvitin 2, respectively. These data for the first time documented on a large scale the major states and sites of phosphorylation of phosvitins with a total of 151 phosphorylation sites. Importantly, the present work also provided the first direct de novo protein amino-acid sequence data for phosvitin 1 protein and evidence for the full expression of vitellogenin I gene.

Biological significance
We have for the first time generated a large number of phosphopeptides (~100) and identified 151 phosphorylation sites of phosvitin 1 and phosvitin 2, respectively. Importantly, this study also led to the discovery of a novel phosvitin 1 and provided the first direct de novo protein amino-acid sequence data for the full expression of vitellogenin I gene. There is considerable interest in naturally occurring phosphopeptides/phosphoproteins and their application in biomedical fields and in the food industry because of their molecular characteristics and non-toxic nature, hence, our work opens new avenues to pursue such endeavors. In addition, the results provide important fundamental biologic information relevant to evolutionary developments of vertebrate animals beginning with the earliest fish, reptiles, birds and more contemporary mammals. For instance, the

Abbreviations: PTH, parathyroid hormone; DTT, dithiothreitol; RP-HPLC, reverse-phase high-performance-liquid-chromatography; SDS-PAGE, sodium dodecyl sulfate and polyacrylamide gel electrophoresis; OPN, osteopontin; BSP, bone sialoprotein; ECM, extracellular matrix; Ca-P, calcium phosphate; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; mCKII, microsomal casein kinase II; CKI, casein kinase I.

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abundance of phosvitins with a unique degree of phosphorylation in the egg yolk of fish, reptiles, and birds suggests potential biological functions of phosvitins which are critical to the development of embryos of these distant vertebrates.

1. Introduction

Egg yolk phosvitin is a phosphoprotein of ECM origin with a high degree of phosphorylation and possesses biological properties similar to those of ECM phosphoproteins found in mineralized tissues [1–4]. Phosvitin has been shown by classical chemistry approaches to have almost all of its serine residues phosphorylated based on calculations using the amount of inorganic phosphate released by chemical treatment [1]. The extent of phosphorylation is clearly reflected in the biological properties of this phosphoprotein which range from very strong divalent metal binding capacity, particularly iron [2,3], and calcium, and effects on calcium phosphate crystal nucleation [4], as well as antioxidant and anti-bacterial effects [5–7]. The latter antioxidant and anti-bacterial properties, however, are unique to phosvitin and not shared by other ECM phosphoproteins. The suggested high phosphoserine content of phosvitin is reminiscent of our most recent identification of a 22 kDa fish bone phosphoprotein (~60 mol of P-Ser/mol of protein) [8], and that of phosphophoryn, suggested to have over ~90 mol of P-Ser/mol protein [9–11]. The latter is a cleavage product of dentin sialophosphoprotein belonging to the SIBLING family of OPN and BSP whose covalently-bound phosphate groups are directly related to the deposition of calcium phosphate crystals [12]. The non-collagenous bone extracellular matrix (ECM) phosphoproteins are involved in a number of biological events such as the regulation of biomineralization and cellular activity/behavior in normally mineralizing tissues which include bone [13–19], cartilage [20], and dentin [9]. In addition to their involvement in biomineralization and its regulation [18,21–23], bone ECM phosphoproteins are implicated in modulating cellular function and behavior of bone cells such as osteoblasts [24–26] and osteoclasts [27–31], as well as other cells such as tumor and immune [32,33] via promoting cell adhesion, motility and transmembrane signaling. The involvement of both BSP and OPN in such biological functions has been predominantly linked to the presence of integrin receptor binding tripeptide RGD sequence in these proteins which interacts with the cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_1$ [30,34–36]. We have, however, recently demonstrated that the covalently-bound phosphate groups are critically involved in "outside-in" signaling and impact osteoclast differentiation and bone resorption [37]. The phosphorylation states and precise sites of phosphorylation on purified bone OPN and BSP including their topographical distribution have been clearly defined [18,38]. The extent of phosphorylation on each phosphorylation site (10–11 sites) varied between 0.3 and 1.0 mol phosphate/mol of peptide [17–19,38]. These phosphorylation sites and peptide regions defined in those studies were predominantly recognition sequences for casein kinase II (mCKII) [16–18,39]. These studies established that not all of the molecules are phosphorylated on each of the potential phosphorylation sites within a given population of molecules isolated from bone. Further in vivo studies confirmed the "natural variation" of the state of phosphorylation of BSP and OPN as a function of time and new bone development [17].

Unlike OPN and BSP, where complete phosphorylation sites and their distribution have been clearly defined and documented [18,38], there are no such detailed characterizations for phosvitin. The attempts to isolate and characterize phosphopeptides of phosvitin have been very limited and achieved minimal success as only a few phosphopeptides have been partially characterized with poor localization of a few P-Ser/P-Thr residues within the primary amino-acid sequence [40,41]. Most of these attempts were carried out using proteolytic digests followed by isolation and characterization of the peptides by classical approaches using gel filtration and biochemical/chemical analysis. In the present work we have utilized a series of experimental/technical approaches in combination with chemical modifications and mass spectrometry (MS) to identify a large number of phosphopeptides, coupled with extensive localization of phosphorylation sites and their distribution within the chicken egg yolk phosvitin protein sequences. During these studies we have also identified for the first time at a protein level a second form of phosvitin derived from the vitellogenin I protein.

2. Materials and methods

2.1. *SDS-PAGE of chicken phosvitin before and after enzymatic dephosphorylation, base-catalyzed dephosphorylation and enzymatic deglycosylation*

(i) *Enzymatic dephosphorylation of native chicken egg yolk phosvitin.* 30 μg of native phosvitin (Sigma, Co.) was dephosphorylated by 15 units of acid phosphatase (from potato, Sigma, Co.) in 0.2 ml of 50 mM sodium acetate buffer, pH 5.0, at 37 °C overnight. The general procedures were carried out essentially as previously described [17].

(ii) *Base-catalyzed dephosphorylation and derivatization of P-Ser and P-Thr residues by DTT.* Aliquots of phosvitin, 30 μg each, were subjected to: (a) 0.3 M NaOH only for 1 h at 50 °C, and (b) 0.3 M NaOH + 10 mM DTT for 1 h at 50 °C as described previously [38,42,43].

(iii) *Enzymatic deglycosylation of native phosvitin.* Aliquots of native phosvitin 30 μg in each case were treated with: (a) no treatment, (b) 12 m units of neuraminidase (Roche Diagnostics, Co.), (c) 12 mU neuraminidase + 6 mU O-glycosidase (Roche Diagnostics, Co.), (d) 12 mU units neuraminidase + 6 mU O-glycosidase followed by digestion with 3% (w/w) trypsin for 6 h, and a second addition of 3% (w/w) trypsin and further incubated for an additional 18 h, and (e) 12 mU neuraminidase + 6 mU O-glycosidase, followed by additional treatment with 0.3 M NaOH for 1 h at 50 °C. All the treatments