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Calcineurin dephosphorylates the C-terminal region of filamin in an important regulatory site: A possible mechanism for filamin mobilization and cell signaling ☆

Elizabeth García [a], Alfred Stracher [b], David Jay [a,*]

[a] Departamento de Biomedicina Molecular Cardiovascular, Instituto Nacional de Cardiología, Ignacio Chávez, Mexico D.F. 14080, Mexico
[b] Department of Biochemistry, Health Science Center at Brooklyn, State University of New York 11203, USA

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Abstract

Filamin is a phosphoprotein that organizes actin filaments into networks. We report that a purified C-terminal recombinant region of filamin is a suitable substrate for calcineurin in vitro. Furthermore, 1 μM cyclosporin A (CsA), a specific calcineurin inhibitor, reduced the dephosphorylation of the recombinant fragment in 293FT cells. Mutagenesis analysis showed that a dephosphorylation step occurred in Ser 2152, which was previously shown to provide resistance to calpain cleavage when endogenous PKA is activated. In contrast, phosphorylation of Ser 2152 was recently reported to be necessary for membrane dynamic changes. In this regard, we found that CsA protects filamin in platelets from calpain degradation. Results could be combined with available information in a single model, assuming that some of the peptide fragments released by calcineurin-regulated calpain action could mediate actions in downstream pathways, which may help to resolve the controversies reported on the role of filamin phosphorylation in actin dynamics.

Keywords: Androgen receptor; Calpain; Calcineurin; Cyclosporin; Filamin; Insulin receptor; NFAT; Pak; RSK; Tau

Filamin A (FLNa, non-muscle filamin or actin-binding protein-280, ABP-280 or ABP)[1] are homodimers with polypeptide subunit chains of 2647 amino acids, corresponding to 280 kDa. The functional domains of each subunit include an F-actin binding region, a self-association domain, and a membrane glycoprotein binding region [1]. In cells from different origins, including platelets, ABP is an important component of the cytoskeleton, where it promotes rearrangement of actin filaments in response to a variety of physiological and exogenous stimuli [1,2]. Previous studies indicated that a dynamic phosphorylation/dephosphorylation process may modulate the interaction of ABP with other cytoskeletal elements. Dephosphorylation of ABP by treatment with *Escherichia coli* alkaline phosphatase resulted in the loss of its ability to crosslink F-actin into a low-speed sedimentable complex (platelet cytoskeleton) [3,4], whereas phosphorylation of the native protein by cAMP-dependent protein kinase (PKA) strengthens this structural framework by protecting the molecule against proteolytic cleavage by calpain. In this case, the phosphorylation site lies in the 100 kDa carboxy-terminal fragment produced by calpain cleavage [4–7].

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* Corresponding author. Fax: +52 55 5573 0926.
E-mail addresses: david.jay@cardiologia.org.mx, davjaymx@yahoo.com (D. Jay).

[1] *Abbreviations used:* ABP, actin-binding protein or filamin A; AR, androgen receptor; Calp., calpain; CN, calcineurin; CsA, cyclosporin A; EGF, epidermal growth factor; FLNa, filamin A or actin-binding protein; Forsk., forskolin; hsp90, heat-shock proteins of average weight 90 kDa; IBMX, isobutylmethylxanthine; IR, insulin receptor; LIM, for LIN11, ISL1 and MEC3 kinases; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; Pak1 and 6, p21-activated kinase 1 and 6; PKA, cAMP-dependent protein kinase; RSK, ribosomal S6 kinase; SIPP, signaling integrator proteolytic peptide.

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In this regard, FLNa was described as a phosphoprotein whose phosphate content ranges from 18 to 40 mol of Pi/mol FLNa [4]. Indeed, the analysis of the amino acid sequence of FLNa [1] revealed multiple consensus sequences specific for diverse kinases, including three sites for PKA at residues 167, 2152, and 2336, whereas the calpain cleavage site has been localized to residues 1761–1762, 100 kDa away from the C-terminal end. There is an additional calpain site at 10 kDa from the C-terminal region but its cleavage is ineffective. Thus, calpain digestion results in polypeptides of 180, 100, 90, and 10 kDa. From previous studies [8], the threonine at position 167 appears to already be phosphorylated in native ABP, whereas the remaining potential PKA sites appeared to be free in the molecule and confer proteolytic stability upon becoming phosphorylated [4–7].

More recently, in an effort to identify the functionally significant phosphorylation sites in ABP we generated in vitro different C-terminal fragments, which, in combination with site-directed mutagenesis analysis, confirmed Ser 2152 as the only PKA phosphorylation site in the C-terminal region of ABP [9]. Subsequent studies have shown that Ser 2152 phosphorylation is stimulated in vivo after PKA activation [10,11]. The effect and full consequences of phosphorylation of FLNa, however, have remained unclear and to a certain extent controversial. In contrast to the stabilizing effect of phosphofilamin on cytoskeletal structure reported by us [3–6], phosphorylation of FLNa was reported to be necessary for membrane dynamic changes. In fact, Pak1 and RSK were recently found to phosphorylate FLNa in Ser 2152 in processes which involved membrane ruffle formation and EGF-induced migration of human melanoma cells, respectively [12,11]. In addition, we lack conclusive evidence regarding the role of dephosphorylation and how this step can be coordinated with the opposite phosphorylation reaction to regulate FLNa function.

In a previous study, calcineurin was found to be the only calcium an calmodulin-dependent protein phosphatase present in human platelets that, in addition, was able to reduce ^{32}P label on many cytoskeletal proteins, including ABP, from a Triton X-100 lysate of thrombin stimulated platelets [13]. At that time, however, the physiological role of this dephosphorylation reaction remained unclear. Platelet calcineurin was first purified from human platelets by Tallant and Wallace [14].

Calcineurin has been described as a calcium/calmodulin-dependent threonine/serine protein phosphatase required for the dephosphorylation and subsequent translocation of the nuclear factor of activated T cells (NFAT) proteins from the cytoplasm to the nucleus in most immune system cells [15]. As substrates for calcineurin, NFAT proteins are major targets for the immunosuppressive drug cyclosporin A (CsA). Inactivation of calcineurin by CsA, however, can also alter a spectrum of other cellular functions in cells outside the immune system; a process which may underlie, at least in part, the toxic effects of the drug [16,17]. In this report, we provide clear evidence that Ser 2152 in the C-terminal region of FLNa is a suitable substrate for calcineurin in vitro and in vivo and that the calcineurin-specific inhibitor CsA protects in a doses-dependent manner native filamin in platelets from calpain degradation. We propose a mechanism by which FLNa becomes susceptible to calpain degradation after activated calcineurin promotes its dephosphorylation. After degradation, filamin would allow actin cytoskeleton reorganization. In addition, we also propose a model in which the present results, together with available information, may be combined in a single scheme, assuming that some of the peptide fragments released by calcineurin-regulated calpain action could mediate actions in downstream pathways, which would resolve some of the controversies reported on the role of FLNa phosphorylation during actin dynamics with implications to other cellular routs. The possibility that other cytoskeleton-associated proteins, like tau, could function in a similar fashion is discussed.

Materials and methods

Cells and plasmids

Escherichia coli TOP10 (Invitrogen) was used for plasmid propagation and BL21 (DE3) strain was used for bacterial protein expression. 293FT cells were utilized for experiments involving eukaryotic protein expression. Fresh human platelets, obtained from healthy informed volunteers, were prepared following the procedures described before [18]. Plasmids used were pT7-7M1ABP (containing a 1930 nucleotide sequence section of human endothelial cell FLNa corresponding to the coding region for residues 1717–2360) [7] for bacterial protein expression, and the mammalian expression vectors pcDNA4HM-C2 (containing a 2073-bp section of FLNa corresponding to the coding region for residues 1671–2361) and pcDNA4HM-C2* (carrying a missense mutation, $Ser^{2152} \rightarrow Ala$ (S2152A) in the 1671–2361 fragment) [10]. PCR and site-directed mutagenesis protocols for the elaboration of these plasmids have been previously described [9,10]. Restriction enzyme digestions, agarose gel electrophoresis, and plasmid extractions were performed as described by Sambrook et al. [19]. The missense mutation and part of the DNA sequence of the fragments were verified by fluorescent DNA sequencing utilizing a Perkin-Elmer Genetic Analyzer 310 DNA Sequencer.

Growth, maintenance conditions, and transfection of the 293FT cell line

293FT cells were maintained in 75 cm^2 flasks containing 15 ml of advanced Dulbecco's modified Eagles medium (advanced DMEM) (high glucose) supplemented with 3% fetal bovine serum (FBS), 2 mM GlutaMax, 100 U/ml