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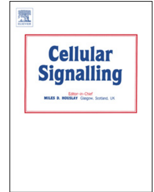

BRI2 and BRI3 are functionally distinct phosphoproteins

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ABSTRACT

Three BRI protein family members have been identified. Among these are BRI3 and BRI2, the latter is associated with Familial Danish and Familial British dementias. 'In silico' sequence analysis identified putative PP1 binding sites in BRI2 and BRI3. This is singularly important, given that protein phosphorylation is a major mechanism regulating intracellular processes. Protein phosphatase 1 (PP1) interacting proteins (PIPs) are fundamental in determining substrate specificity and subcellular localization of this phosphatase. More than 200 PIPs have thus far been reported.
Both BRI2 and BRI3 are type II transmembrane glycoproteins relevant in neuronal systems. Using Myc-BRI2 and Myc-BRI3, wild type and PP1 binding mutant constructs, it was possible to show, for the first time, that in fact BRI2 and BRI3 bind PP1. The complexes BRI2:PP1 and BRI3:PP1 were validated *in vitro* and *in vivo*. The subcellular distribution of BRI2 and BRI3 is similar; both localize to the perinuclear area and Golgi apparatus in non-neuronal cells. However, in SH-SY5Y cells, BRI2 and BRI3 could also be detected in elongated cellular projections ('processes') and in rat cortical neurons both are broadly distributed throughout the cell body, neuritis and the nucleus. Consistently, co-localization of BRI2 and BRI3 with PP1 was evident. The functional significance of these complexes is apparent given that both BRI proteins are substrates of PP1, thus simultaneously this is the first report of BRI2 and BRI3 as phosphoproteins. Moreover, we show that when BRI2 is phosphorylated a significant increase in neuronal outgrowth and differentiation is evident.
Interestingly, the Alzheimer's amyloid precursor protein (APP), forms a trimeric complex composed of PP1 and Fe65, with PP1 having the capacity to dephosphorylate APP at Thr668 residue. The emerging consensus appears to be that PP1 containing complexes are crucial in regulating signaling events underlying neuropathological conditions.

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1. Introduction

Reversible protein phosphorylation is a major mechanism controlling key intracellular events in eukaryotic cells. In fact, recent phosphoproteomic studies have indicated that more than 70% of all eukaryotic cellular proteins are regulated by protein phosphorylation occurring mainly at serine (ser), threonine (thr) and tyrosine (tyr) residues [1]. Protein phosphatase 1 (PP1) is a ubiquitous serine/threonine phosphatase, estimated to dephosphorylate about a third of all proteins in eukaryotic cells [2,3]. In mammals there are four PP1 isoforms: PP1α, PP1β/δ and the splice variants PP1γ1 and PP1γ2 encoded by three different genes *PPP1CA*, *PPP1CB* and *PPP1CC*, respectively [2]. PP1 isoforms are extremely similar in their sequences and the differences are located mainly at the N- and C-terminals. However, they possess specific distinct tissue and subcellular distributions. PP1α, PP1β/δ and PP1γ1 are widely expressed across mammalian tissues, particularly in the brain and PP1γ2 is testis enriched [4–7]. PP1 is a key protein, functionally diverse, involved in processes including a regulatory role in gene transcription, cell cycle regulation, protein targeting and intracellular processing [2,8,9]. The functional versatility and specificity of PP1 relies on the interaction of its catalytic subunit with many different specific regulatory subunits also known as PP1 interacting proteins (PIPs) [10–12]. These control PP1 subcellular localization and determine substrate specificity and activity [2,4,8–10, 13–16]. PIPs interact with the PP1 catalytic subunit (PP1c) through several PP1 binding motifs such as the RVxF, SILK and MyPhone domains [17,18]. Most PIPs interact with PP1c through the well-conserved domain, the RVxF motif. This motif has the consensus sequence [R/K]-$X_{A(0-1)}$-[V/I]-X_B-[F/W], where X_A is any amino acid and X_B is any amino acid except proline [19]. Recently, other consensus sequences have been proposed for the RVxF motif: [HKR]-[ACHKMNQRSTV]-V-[CHKNQRST]-[FW] and [KRL][KRSTAMVHNQ] [VI]{FIMYDP}[FW] [17, 20]. Given that the regulatory subunits control the specificity and the

Abbreviations: PP1, protein phosphatase 1; YTH, yeast-two hybrid; IP, immunoprecipitation; OA, okadaic acid; TM, transmembrane; PIP, PP1 interacting protein.

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diversity of PP1 activity, the key to fully understanding PP1 function lies in studying these regulatory subunits as well as their cellular functions. Yeast two-hybrid (Y2H) screens, of a human brain cDNA library, using the three PP1 isoforms (PP1α, PP1γ1 and PP1γ2) as baits were used, and several PIPs were described including novel PP1 binding partners [21,22]. One of the novel putative PP1 interacting proteins identified, in these screens, was BRI3 which belongs to the BRI family of proteins [22,23]. The *BRI* gene family comprises at least three members, *BRI1*/*ITM2A*, *BRI2*/*ITM2B* and *BRI3*/*ITM2C*, which encode three type II transmembrane glycoproteins BRI1, BRI2 and BRI3, respectively. These three genes have homologs in chimpanzee, Rhesus monkey, cow, mouse, rat and humans, and their genomic organization consists of six exons and five introns [24,25]. The pattern of expression of these three members is quite different across different tissues [25–27]. BRI1, is a 263-residue protein, expressed in several mesenchymal tissues and was found to be most abundantly expressed in chondro- and osteogenic tissues, in thymus and skeletal muscle [26,28–30]. Data suggests a possible involvement of BRI1 in chondro- and osteogenic differentiation [26,29]. The second BRI family member, BRI2, is a 266-residue protein ubiquitously expressed protein, with high levels of expression in the brain, placenta, pancreas, heart, kidney and liver. In the brain, the hippocampus and cerebellum are the regions with the highest mRNA expression levels for BRI2 [25,27,30]. The precise biological function of BRI2 remains unknown. However, some reports indicate that it might be involved in the plasticity of neuronal processes and in the promotion of neuritic outgrowth [31]. It has also been suggested that BRI2 may be involved in apoptosis and in signal transduction, acting as a cell surface receptor since it forms disulfide-linked homodimers that occur at the cell surface. Moreover, several studies demonstrated that BRI2 is post-translationally modified by N-glycosylation at asparagine 170, affecting its trafficking through the secretory pathway to the cell surface [27,30,32,33]. Additionally, bioinformatic analysis of the BRI2 sequence, suggests several putative phosphorylation sites, having only confirmed 4 phosphoresidues (Ser8, Tyr76, Tyr112 and Tyr165) [34–36]. The third family member, BRI3, is a protein composed of 267 amino acids, it is mainly expressed in the brain with the highest levels of expression in the cerebral cortex, medulla oblongata, amygdale, hippocampus, thalamus, striatum, caudate nucleus and spinal cord [25,37]. This protein is also expressed in plasmocytoid dendritic cells, appendix, peripheral blood leukocytes, bone marrow and fetal liver [38]. BRI3's biological function remains to be fully elucidated, although it has been suggested that it may play a role in TNF-induced cell death and in neuronal differentiation [39,40]. The sequence of BRI3 predicts a single site of N-glycosylation at Asn169 and several phosphorylation sites; 4 have already identified (Ser5, Ser30, Thr32 and Thr37) [35,41–46].

BRI2 undergoes regulated intramembrane proteolysis (RIP). First, it is cleaved by a furin and related proteases in the ectodomain, generating a C-terminal 23-residue peptide (3 kDa). Additionally the remaining membrane bound N-terminus, undergoes processing by ADAM-10 and a peptide containing the BRICHOS domain (25 kDa) is released. The resulting membrane associated N-terminal fragment (NTF; 22 kDa) undergoes intramembrane cleavage by SPPL2a/2b to produce a small, secreted, BRI2 C-terminal domain and an intracellular domain (BRI2ICD; 10 kDa), that can translocate to the nucleus [25,47–49]. BRI3 is also processed by a furin, in the ectodomain, resulting in the secretion of a C-terminal 23-residue long peptide [41]. However, although BRI3 is highly homologous to BRI2, it seems that it fails to undergo shedding by ADAM-10 as well as intramembrane proteolysis by SPPL2a/b [41,50]. With respect to BRI1 processing, very little is known, although by aligning the BRI family members one would predict that BRI1 can be cleaved by furin or another related proprotein convertase [41]. To date, the precise biological functions and the proteolytic processing pathways of BRI family protein members have not been fully elucidated.

In the present study by *in silico* analysis the RVxF motif was identified in all the BRI family protein members, signifying that BRI1, BRI2 and BRI3 might be regulated by protein phosphorylation. Given that BRI1 is chondro- and osteogenic tissue specific while the other two proteins, BRI2 and BRI3, are ubiquitous and brain specific, respectively, the work here presented focused on BRI2 and BRI3. Consequently, the putative complexes BRI2:PP1 and BRI3:PP1 were validated, using several *in vitro* and *in vivo* techniques, namely blot overlay assay and co-immunoprecipitations. Furthermore, we established that the KVTF and KISF motifs are the PP1 binding motifs responsible for the interaction between the phosphatase and BRI2 and BRI3 respectively. The functional relevance of the novel complexes was pursued and it was possible to establish that both BRI2 and BRI3 are novel PP1 substrates and both can be dephosphorylated by PP1 *in vitro*. Additionally, BRI2 is important for neuronal differentiation, particularly for neuronal outgrowth that is regulated by protein phosphorylation.

2. Material and methods

2.1. Antibodies

The following primary antibodies were used: mouse monoclonal BRI2 C-8 (Santa Cruz Biotechnology, Inc.), that recognizes the N-terminal of BRI2; rabbit polyclonal BRI3 (Abcam), raised against amino acids 110–258 of human BRI3; rabbit polyclonal CBC2C and CBC3C, that recognizes the C-terminal of PP1α and PP1γ, respectively [4]; rabbit polyclonal APP (Cell Signaling), that recognizes the C-terminal of amyloid precursor protein; and Myc-tag antibody (Cell Signaling), that recognizes myc-fusion proteins. The secondary antibodies used were horseradish peroxidase-conjugated anti-mouse and anti-rabbit (GE Healthcare) for enhanced chemiluminescence (ECL) detection, and Alexa 594-conjugated anti-mouse IgG and Alexa 488-conjugated anti-rabbit IgG (Molecular Probes) for co-localization analysis.

2.2. Expression vectors and DNA constructs

pCMV-BRI2 and pCMV-BRI3 were obtained from the I.M.A.G.E. consortium (MRC Geneservice, UK) and they correspond to BRI2 (GenBank Accession: BC016148) and BRI3 (GenBank Accession: BC098563) cDNAs, respectively, inserted into the mammalian expression vector pCMV-SPORT6. Full-length BRI2 and BRI3 were further subcloned into two different vectors: pET-28c vector (Novagen) and the mammalian expression vector pCMV-Myc (Clontech) to obtain myc-fusion proteins. BRI2 and BRI3 PP1 binding (PP1BM) mutants [17] were generated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The BRI2 F6A (KVTA; BRI2 single mutant), BRI2 V4AF6A (KATA; BRI2 double mutant), BRI3 F6A (KISA; BRI3 single mutant) and BRI3 K3AF6A (AISA; BRI3 double mutant) mutants cDNAs were generated using pCMV-Myc vectors containing wild-type BRI2 and BRI3 with appropriate primers (Table S1). All constructs and mutations were confirmed by DNA sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosystems).

2.3. Brain dissection

Wistar Hannover rats (9–12 weeks) were obtained from Harlan Interfaune Ibérica, SL. All experimental procedures observed the European legislation for animal experimentation (2010/63/EU). No specific ethics approval under EU guidelines was required for this project, since the rats were only euthanized, by cervical stretching followed by decapitation, for brain removal. This is within the European law (Council Directive 86/609/EEC) and during this procedure we took all steps to ameliorate animal suffering and used the minimum number of animals possible. The procedures were approved and supervised by our Institutional Animal Care and Use Committee (IACUC): Comissão Responsável pela Experimentação e Bem-Estar Animal (CREBEA). Briefly, animals were sacrificed by cervical stretching followed by decapitation and both cortex and hippocampus were dissected out on ice. Tissues were further weighed and homogenized on ice with a Potter-Elvehjem tissue homogenizer with 10–15 pulses at 650–

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