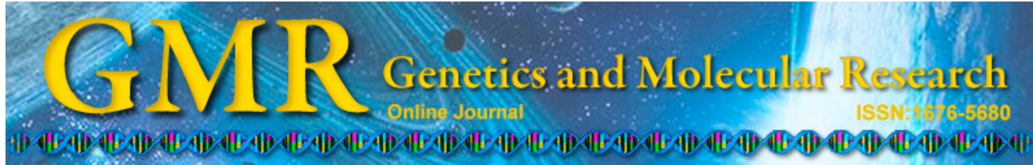

Cloning and functional identification of a novel *BCA3* splice

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ABSTRACT. The human breast cancer-associated gene (*BCA3*) was first discovered in breast and prostate cancer cells lines. *In vivo* studies have shown that BCA3 is mainly expressed in breast tumor cells and not in normal breast and prostate tissues. To date, 3 splice variants of *BCA3* have been reported: a double-absent variant lacking exon 3 and exon 5 (*BCA3-1*), an exon 3-absent variant (*BCA3-2*), and full-length *BCA3*. In this study, we investigated whether a novel *BCA3* splice variant exists that lacks only the exon 5-encoding sequence. *BCA3* variant splices were subcloned and sequenced using reverse transcription-polymerase chain reaction. The preliminary biological functions of the splices were identified using confocal microscopy and a luciferase assay. The absence of exon 3 and exon 5 influenced the subcellular localization of BCA3 and nuclear factor kappa B (NF-kB)-dependent gene expression. Exon 3 and exon 5 of *BCA3* may function together to provide a nuclear localization signal or transport sequence to enter the nucleus, and exon 3 may contain specific sequence(s) or domain(s) that influence the NF-κB signal cascade. The discovery of

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novel *BCA3* splicing indicates a new cancer research area, which may increase the understanding of cancer generation and development.

Key words: *BCA3*; Splice; Nuclear retention; NF-κB

INTRODUCTION

Human breast cancer-associated gene (*BCA3*) was discovered in mRNA screens of breast and prostate cancer cell lines. Further studies, such as those involving immunohistochemistry, revealed that BCA3 was expressed mainly in breast tumor cells *in vivo*, but not in normal breast and prostate tissues that show low-level expression (Kitching et al., 2003; Leon and Canaves, 2003). BCA3 has also been referred to as KyoT2 binding protein 1 (Li et al., 2002; Qin et al., 2004; Qin and Han, 2004) or protein kinase A (PKA)-interacting protein (Sastri et al., 2005). The genomic sequence of human *BCA3* was identified as open reading frame 17 on chromosome 11, is composed of 1319 base pairs, and encodes 210 amino acids. It is highly conserved among mammals and shares 70% identity with the mouse protein sequence (79% with conservative substitutions).

BCA3 has been reported to bind the nuclear factor kappa B (NF-kB) p65 subunit and regulates NF-kB transcriptional activity (Gao et al., 2006, 2008a). NF-kB regulates the transcription of many genes involved in immunoinflammatory responses, cell proliferation, and survival, and thus plays crucial roles in the pathogenesis of many diseases. Importantly, NF-kB is involved in many cellular processes (Burleigh and Woolsey, 2002), including interleukin (IL)-1b production (Petersen et al., 2005), invasion (Hall et al., 2000), and inhibition of apoptosis of parasite-infected cells (Petersen et al., 2006). BCA3, the catalytic subunit of PKA (PKAc), and the p65 subunit of NF-kB form a cytosolic complex that may be modulated through PKA activators. Phosphorylation of p65 by PKA is essential for its translocation. Overexpression of either BCA3 alone or concomitantly with the amino terminus of PKAc (CAT 1-29), which blocks the interaction between PKAc and BCA3, results in constitutive localization of p65 in the nucleus, indicating that BCA3 regulates the rate at which p65 enters the nucleus (King et al., 2011).

Recently, Gao et al. (2006) reported that BCA3 is a NEDD8 substrate. Neddylation is the process by which the protein neural precursor cell-expressed and developmentally downregulated gene *NEDD8* is covalently linked to proteins in a process similar to ubiquitination. Neddylation of BCA3 suppresses NF-kB-dependent transcription by enabling binding of neddylated BCA3 to the NF-kB transcription factor p65 and the cyclin D1 promoter.

In 2003, 3 proline-rich splices of *BCA3* and their possible functional domains were identified using reverse transcription-polymerase chain reaction (RT-PCR) analysis (Kitching et al., 2003), including 2 putative splice variants of *BCA3* in which exon 3 or both exons 3 and 5 were spliced out. Indications of the potential *in vivo* functions of BCA3 can be derived from the positions of consensus protein phosphor-binding sites encoded by particular exons. All 3 alternative *BCA3* transcripts harbor exon 4, which encodes 2 of 5 consensus SH2 binding site sequences. Exon 6 is also conserved in the 3 variants and encodes 3 contiguous consensus SH2 binding sequences. In addition, 2 of the 3 consensus protein kinase C (PKC) phosphorylation motifs (exons 1 and 6) and one of the 2 consensus casein kinase 2 (CK2) phosphorylation sites (exon 4) are present in all *BCA3* variants. Exon 3 is absent from both alternative transcripts, and splicing out of exon 3 removes the overlapping consensus sequences for CK2, PKC, and a tyrosine kinase consensus sequence site. Similarly, loss of exon 5 in the smallest *BCA3* variant removes the encoded consensus sequence for a cAMP kinase phosphorylation site. Furthermore, a major binding determinant for PKA is located

in exons 5 and 6 of *BCA3*. This region possesses a putative PKAc phosphorylation site and thus may facilitate the increased binding observed between PKAc and BCA3 (Gao et al., 2006).

In this study, we used RT-PCR to clone and sequence 4 alternatively spliced *BCA3* transcripts. Using bioinformatic analysis, we identified a novel *BCA3* splice that lacks only the exon 5 coding sequence. Exon 5 is thought to contain the encoded consensus sequence for a cAMP kinase phosphorylation site and to constitute the major binding determinant for PKA. We used confocal microscopy and a luciferase assay to determine the biological function of the novel splice variant.

MATERIAL AND METHODS

Cell lines

The human normal liver cell lines LO2 and HL7702 were obtained from the Pre-clinical Medicine College of Nanjing University of Chinese Medicine and were maintained in 5% CO_2 at 37°C in Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies; Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The hepatocarcinoma cell line (HepG2), ovarian cancer cell line (SKOV3), epithelial adenocarcinoma cell line (HeLa), and human renal embryonic cell line (293T) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS (Gibco). Hybridoma cells secreting 4C13 monoclonal antibody and SP2/0 cells were maintained in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% (v/v) FBS. All cell lines were maintained at 37°C in the presence of 5% CO_2.

Cloning of human *BCA3*

BCA3 genes from LO2, HL7702, HepG2, SKOV3, and HeLa cells were amplified by reverse transcription (RT Kit; Promega; Madison, WI, USA) and PCR using an upstream and downstream primer pair according to published human *BCA3* region sequences (Gene ID: 56672). PCR products were separated by agarose gel electrophoresis. Each sample showing a predicted molecular weight (MW) was purified, cloned into the pGEM-Teasy vector (Promega), and identified by sequence analysis.

Mammalian expression of *BCA3* mutants

BCA3 mutant genes were respectively inserted into the pEGFP-N1 vector (Invitrogen; Life Technologies) using restriction enzyme digestion and ligation. Recombinant plasmids were transfected into 293T cells using a standard method with Lipofectamine 2000 Reagent (Gibco). Briefly, 293T cells were grown on a 6-well plate at 3 x 10^5 cells/well in complete medium overnight at 37°C, and then respectively transfected with *BCA3* mutant plasmids. After an additional 48 h of incubation, the cells were collected and observed using confocal microscopy.

Transient luciferase assay

Two hundred and ninety three 29 T cells were cultured on 24-well plates and transfections were conducted with double-luciferase reagent (Promega). For each transfection, 100 ng or 200 ng NF-kB_LUC were used, as well as *BCA3* or its mutant plasmid. Fifty nanograms ng GAL4-TK-expressing *Renilla* luciferase was set as an internal control. Twenty-four hours after transfection, cells were stimulated with 10 ng/mL tumor necrosis factor alpha (TNF-α) for 30 min for the luciferase

assay. Luciferase activity and transfection efficiency were normalized based on *Renilla* luciferase activity. All experiments were performed in triplicate and repeated at least 2 times.

RESULTS

Cloning and sequencing of novel *BCA3* splices in different human cell lines

BCA3 splices were amplified by RT-PCR in LO2, HL7702, HepG2, SKOV3, and HeLa cells. As shown in Figure 1A, specific *BCA3* genes could be observed only in SKOV3 and HeLa cells. Each of the separated lines was sequenced, and a novel variant lacking only exon 5 was discovered and was designated as *BCA3-3* (Figure 1B). This splice has not been reported previously, possibly because it has a similar molecular weight to the exon 3-absent variant (*BCA3-2*).

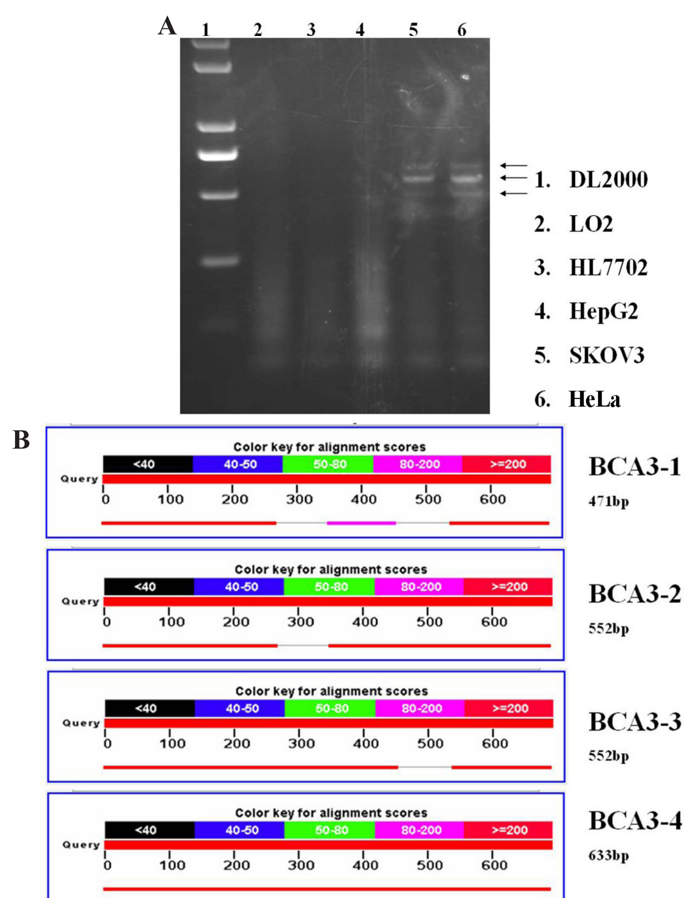

Figure 1. Cloning and sequencing of the BCA3 genes in different human cell lines (normal liver cell lines LO2 and HL7702, hepatoma cell line HepG2, ovarian cancer cell line SKOV3 and Epithelial Adenocarcinoma cell line HeLa). **A.** Agarose electrophoresis identification of RT-PCR products using BCA3 up- and downstream primers. Cells were lysed in TRIzol and total RNA was distilled. The products of RT-PCR were purified, cloned into pGEM-T-easy vector and identified by sequence analysis; **B.** Nucleotide sequences of different BCA3 splices determined by DNA sequencing, including the variant in which exon 5 was spliced out (BCA3-3). It had similar molecular weight with exon 3-absent variant (BCA3-2).

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Absence of exon 5 in *BCA3* may influence its cellular localization

The genes of 4 *BCA3* splice variants were respectively cloned and ligated to pGEFP-N1 [containing a green fluorescence protein (GFP) region] to generate expression vectors (Figure 2A). Each vector was transfected into 293T cells using a standard transfection method. Two days later, the cells were collected and observed by confocal microscopy. The cells stained for BCA3-GFPs (Figure 2B, left column), nuclei (Figure 2B, middle column), and the 2 images merged (Figure 2B, right column), indicating that the absence of exon 3 and/or exon 5, induced translocation of BCA3 from a whole-cell distribution to a perinuclear or nuclear location (BCA3-1, 2, and 3).

Novel *BCA3* splice (*BCA3-3*) may inhibit NF-κB-dependent gene expression

Whereas 10 ng/mL TNF-α stimulation induced NF-kB activation by 3-fold compared to the control sample, and co-transfection of *BCA3* (*BCA3-4*) further enhanced the transcriptional activity by at least 3-fold, in *BCA3* mutant-transfected samples (*BCA3-1*, *2*, *3*), NF-kB activation was inhibited (Figure 3). This indicates that NF-kB-dependent gene expression normally requires BCA3 under physiological conditions, and that the spliced fragments encoded by exon 3 and/or exon 5 are crucial for this process.

DISCUSSION

BCA3 was initially identified in breast cancer cells (Kitching et al., 2003) and is known to play several distinct and important roles. It can interact with and enhance the role of PKA/NF-kB in the nucleus, where it facilitates the nuclear translocation of PKAc (Sastri et al., 2005). BCA3 has also been shown to be a novel p65-interacting protein. It appears to serve as a molecular bridge between p65 and PKAc and acts to scaffold PKAc to NF-kB in the cytosol, thereby promoting their interaction and the subsequent p65 phosphorylation at Ser-276, which in turn promotes the nuclear translocation of p65 to enhance NF-kB-mediated gene expression (Gao et al., 2008b). Furthermore, the variability and multiple modifications of the amino terminal region of PKAc strongly suggest that it is involved in substrate recognition, localization, or targeting (Colledge and Scott, 1999; Shabb, 2001).

BCA3 binds to the amino terminus (residues 1-39) of PKAc, an interaction that is localized to the A helix (residues 14-39) of PKAc and the carboxyl terminus of BCA3. The N-terminus is a genetically diverse region of the protein that precedes the conserved core and targets the protein to distinct subcellular locations through myristylation, phosphorylation, and deamidation (Carr et al., 1982; Yonemoto et al., 1993; Guthrie et al., 1997; Gangal et al., 1999; Pepperkok et al., 2000).

BCA3 can regulate the mode of PKA signaling in the NF-kB cascade based on its expression level. In cell lines in which BCA3 expression is low, elevation of cAMP can decrease p65-PKA binding and p65 phosphorylation at Ser-276, eventually leading to downregulation of NF-kB-dependent transcription. Moreover, activation of the PKA cascade by upregulating the intracellular cAMP level promotes cancer cell apoptosis by antagonizing the NF-kB activation cascade. By contrast, in high BCA3-expressing cancer cells, such as MDA-MB231, MCF7, or BCA3-overexpressing 293 cells, PKA-activating agents can increase p65-PKA binding, p65 phosphorylation, and upregulate NF-kB-dependent transcription, particularly in anti-apoptotic genes, thus contributing to NF-kB-dependent cell survival.

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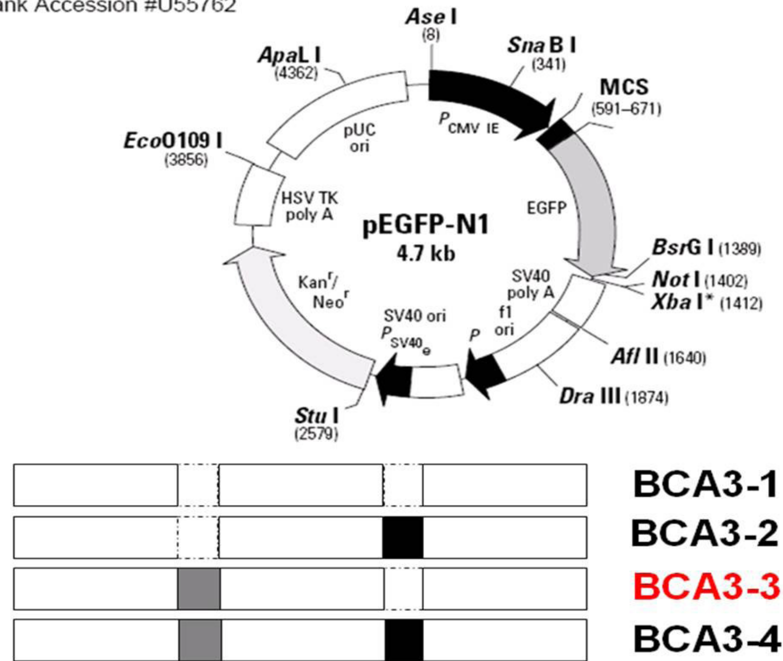

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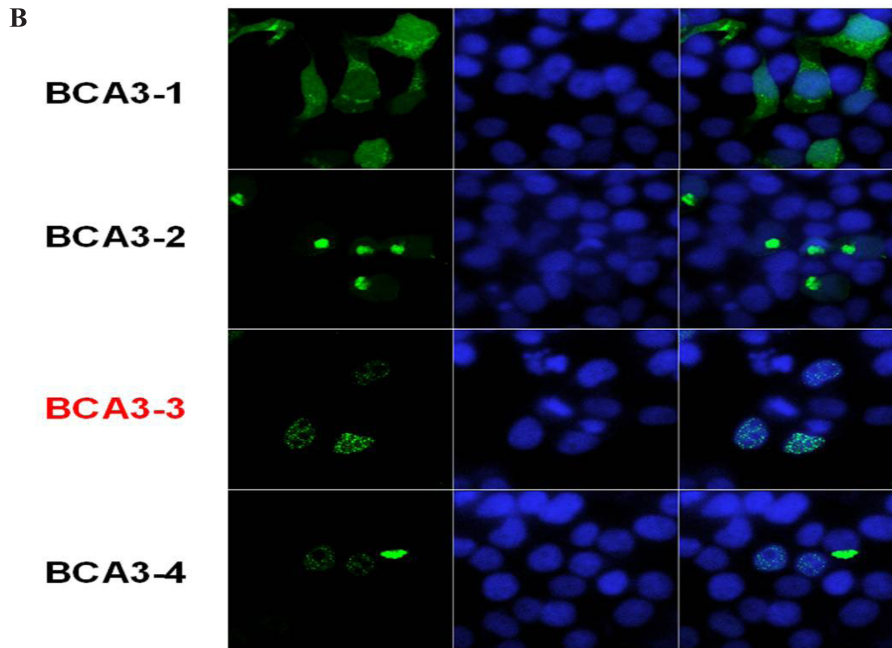

Figure 2. A. Localization of BCA3 splices in 293T cells. **B.** Confocal microscopic images stained for BCA3-GFPs (green; left column), nuclei (blue; middle column), and the two images merged (right column). Exon-absent induces translocation of BCA3 from a whole-cell to a perinuclear (BCA3-2) or nuclear location (BCA3-3).

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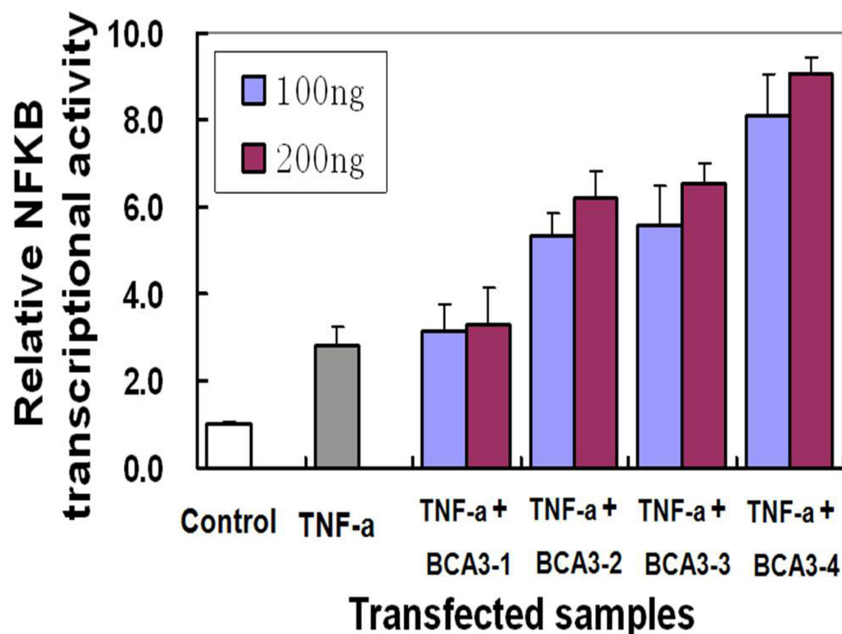

Figure 3. Inhibition of the NF-κB-dependent gene expression by BCA3 mutants. Two hundred and ninety three T cells were transfected with plasmids encoding BCA3 or its mutant gene together with GAL4-TK, stimulated with 10 ng/ml TNF-α, and 30 minutes later, the cell lysate was obtained for the luciferase assay. Either in 100 or 200 ng plasmid transfected samples, BCA3 mutants showed decreased luciferase activity.

In 2003, Kitching et al. (2003) identified 3 proline-rich splice variants and predicted several putative functional domains of BCA3, suggesting that there were 2 mutants of *BCA3*: *BCA3-1* (double-absent variant lacking exon 3 and exon 5) and *BCA3-2* (exon 3-absent variant). In this study, we cloned and sequenced a novel *BCA3* splice variant, *BCA3-3*, which lacks only the exon 5-encoding sequence. Exon 5 was found to encode a consensus sequence for a cAMP kinase phosphorylation site as well as a major binding determinant for PKA. In addition, we used confocal microscopy and a luciferase assay to examine the preliminary biological function of the novel splice; the results indicated that the absence of either exon 3 or exon 5 could influence the subcellular localization of BCA3, and thus related NF-kB-dependent gene expression.

BCA3 is localized to the nucleus where it is known to interact with the catalytic subunit of PKA (PKAc). BCA3 acts as a novel protein that interacts with the 30 N-terminal residues of PKAc; it most likely contributes to the integration of PKA signaling by retaining the C subunit in the nucleus. It is a molecular scaffold that simultaneously binds PKAc and NF-kB p65 in the cytosol; thus, disruption of this complex alters the rate at which NF-kB enters the nucleus. Phosphorylation of p65 by PKAc is prevented by BCA3 in the cytosol, allowing p65 to translocate into the nucleus at a faster rate. However, BCA3 can be localized in interfibrillary mitochondria and is upregulated in the cardiac mitochondrial subpopulation during ischemic injury. Mitochondria isolated from *BCA3* gene-transferred hearts showed increased mitochondrial localization of BCA3, decreased reactive oxygen species generation, enhanced calcium tolerance, decreased mitochondrial cytochrome C release, and enhanced

phosphorylation of mitochondrial PKA substrates under ischemic stress conditions. Furthermore, upregulation of *BCA3* had a protective effect on ischemic injury by enhancing mitochondrial integrity. Overexpression of both BCA3 and TAp73 resulted in their association in the mitochondria, leading to cell death (Leung and Ngan, 2010). BCA3 can interact with the mitochondrial-localized apoptosis-inducing factor (AIF) under both normal conditions and oxidative stress. Furthermore, BCA3 is a novel Rac1-interacting partner in osteoclasts and binds directly to Rac and GTP-Rac, but not to GDP-Rac, *in vivo*.

BCA3 contains several motifs, including the carboxyl terminal region, which harbors a potential PKA phosphorylation site and a PDZ-binding motif. Domain mapping studies of BCA3 indicate that the BCA3 C-terminal region binds to PKAc (Sastri et al., 2005) and p65, and that these interacting regions on the BCA3 molecule are likely distinct because BCA3 protein simultaneously binds both PKAc and p65 and enhances the PKAc-mediated phosphorylation of p65. The BCA3 N-terminus, which contains the nuclear-localizing sequence (amino acids 15-21) that accounts for its nuclear localization (Sastri et al., 2005), is responsible for the retention of p65 in the nucleus. In addition to the consensus phosphorylation sites, the carboxyl terminus of BCA3, -F-P-V, corresponds to the consensus binding sequence, -F/Y-x-(A/F/V), derived from Type II PDZ domain-containing proteins (Songyang et al., 1997; Ligensa et al., 2001). PDZ domain proteins are typically localized at the cytoplasmic face of the cell membrane where they bind to the carboxyl termini of their substrates, and in many cases contain additional kinase domains (Ponting et al., 1997; Carraway and Sweeney, 2001; Ligensa et al., 2001).

Here, the shortest BCA3 splice showed subcellular localization that was distinct from that of full-length BCA3 (BCA3-4), suggesting that exon 3 and exon 5 together confer a nuclear localization signal (NLS) or transport sequence to enter the nucleus, as the absence of both exon 3 and exon 5 induced distribution throughout the cell. Moreover, the shortest splice of BCA3 (BCA3-1) and BCA3-2 showed weaker upregulation activity for NF-kB-dependent gene expression relative to BCA3-4, mainly because of the absence of the major binding determinant for PKA encoded by exon 5. Similarly, *BCA3-3*, the novel splice cloned from SKOV3 and HeLa cancer cells, showed exhibited inhibitory activity, suggesting that exon 3 has an unknown specific sequence(s) that influences the NF-kB signal cascade.

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