Short Communication

Breast cancer-associated protein – a novel binding partner of Mason-Pfizer monkey virus protease

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We identified breast cancer-associated protein (BCA3) as a novel binding partner of Mason-Pfizer monkey virus (MPMV) protease (PR). The interaction was confirmed by co-immunoprecipitation and immunocolocalization of MPMV PR and BCA3. Full-length but not C-terminally truncated BCA3 was incorporated into MPMV virions. We ruled out the potential role of the G-patch domain, a glycine-rich domain located at the C terminus of MPMV PR, in BCA3 interaction and virion incorporation. Expression of BCA3 did not affect MPMV particle release and proteolytic processing; however, it slightly increased MPMV infectivity.

Synthesis of the Mason-Pfizer monkey virus (MPMV) polyprotein precursors Gag, Gag–Pro, and Gag–Pro–Pol requires two ribosomal frameshift events. gag encodes the structural proteins matrix, phosphoprotein p12, capsid (CA), nucleocapsid and p6; pro encodes dUTPase and protease (PR); and pol encodes reverse transcriptase and integrase. MPMV encodes a unique sequence called the G-patch domain (GPD). During maturation of the Gag–Pro polyprotein, the GPD remains attached transiently to the C-terminal part of the PR (PR17), from which it is later detached by PR itself, yielding GPDPR13. Both PR17 and GPDPR13 are present in released virions, and display similar proteolytic activity and substrate specificity (Zábranská et al., 2007; Zábranský et al., 1998). Although the role of the GPD in the MPMV life cycle has been studied (Křižová et al., 2012; Svec et al., 2004; Zábranská et al., 2007), its precise function remains unclear.

To analyse whether the GPD interacts with any cellular proteins, we performed yeast two-hybrid screening with catalytically inactive MPMV PR17(D26N) as bait against proteins expressed from a HeLa cDNA library (Clontech). The transformants were screened on a selective medium deficient in adenine, His, Leu and Trp, followed by further selection on a medium containing X-a-Gal. Twenty colonies with the highest β-galactosidase activity were selected for rescoring, and six clones were sequenced and analysed by BLAST. One of the positive clones encoded breast cancer-associated protein (BCA3). The full-length BCA3 protein, also known as AKIP1 (A-kinase-interacting protein 1), consists of 210 aa encoded in five of six exons (Fig. 1a) with translation initiation in exon 2 (Kitching et al., 2003). The amino acid sequence comparison of human BCA3 with orthologues from different mammalian species revealed conserved segments (Table S1, available in the online Supplementary Material). BCA3 was first identified and characterized in human breast and prostate cancer cell lines (Burger et al., 1998; Kitching et al., 2003; Sastri et al., 2013). Several BCA3 splice variants have been identified in various cell lines (Kitching et al., 2003; Sastri et al., 2005). Although BCA3 has been reported to interact with various cellular proteins, its precise cellular role remains unclear. BCA3 was identified as a binding partner of the catalytic subunit of cAMP-dependent protein kinase A (PKA), causing elevated PKA accumulation in the nucleus (Sastri et al., 2005). BCA3 was also shown to interact with the p65 subunit of the nuclear factor NFkB, and to serve as a bridge between p65 and the catalytic subunit of PKA, promoting their interaction (Gao et al., 2008, 2010). In one report, BCA3 was shown to repress NFkB-dependent transcription by recruiting the histone deacetylase SIRT1 in a

One supplementary table is available with the online version of this paper.
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(c)
neddlyation-dependent manner (Gao et al., 2006). Another study showed that BCA3 enhanced NFκB-dependent transcription (Gao et al., 2008, 2010). BCA3 has also been shown to interact with p73, a p53 tumour suppressor gene homologue involved mainly in apoptosis (Leung & Ngn, 2010). Recently, BCA3 was shown to be upregulated in cardiac myocytes in response to oxidative stress (Sastri et al., 2013).

To verify that the yeast two-hybrid screening results reflected a direct interaction between BCA3 and MPMV PR17 in intact HEK-293 cells, we performed co-immunoprecipitation followed by Western blot analysis. As active MPMV PR is cytotoxic, we prepared a construct c-myc-PR17(D26N), encoding inactive MPMV PR (PR17) (carrying a D26N mutation) with N-terminal fusion to a c-myc tag. To analyse whether the GPD of MPMV PR17 could serve as a BCA3-binding target, we prepared a vector encoding the PR variant ΔGPDPR13(D26N) lacking the GPD. The full-length gene encoding human BCA3 was obtained by reverse transcription of RNA isolated from HeLa cells. It was then cloned into pCMV vector (Clontech) with N-terminal fusion to a haemagglutinin (HA)- or c-myc tag. HEK-293 cells were co-transfected with vectors encoding c-myc-tagged MPMV PR and HA-tagged BCA3. The expression of MPMV PR17, PR13 as well as BCA3 was verified by Western blotting (Fig. 1b, upper panels) using monoclonal anti-HA peroxidase conjugate clone HA-7 (Sigma-Aldrich) and monoclonal anti-c-myc antibody (9E10; Santa Cruz).

Immunoprecipitation with anti-c-myc antibody (9E10) was carried out at 24–48 h post-transfection. Complexes of BCA3 co-migrated with the bulk of MPMV CA, with a peak at a sucrose density of 1.14–1.16 g ml⁻¹ (fractions 5 and 6), which corresponds to the buoyant density of MPMV. To exclude that incorporation of BCA3 into MPMV particles is due to overexpression of BCA3, we strove to identify whether endogenously expressed BCA3 is also packed into MPMV virions. However, we failed to detect endogenous levels of BCA3 in selected cell lines (COS-1, HeLa, MCF7, MDA, HEK293, Jurkat) using several commercial antibodies. Reverse transcription quantitative (q) PCR revealed a very low amount of BCA3 RNA in all of the above-mentioned cell lines (data not shown).

Fig. 1. Amino acid sequence alignment of BCA3 from different species, and interaction of MPMV PR and BCA3. (a) Boxes above the aligned sequences indicate BCA3 exons 2–6. (b) Co-immunoprecipitation of MPMV PR17 and ΔGPDPR13 with BCA3. HEK-293 cells were transfected with vectors expressing MPMV c-myc-PR17(D26N), c-myc-ΔGPDPR13(D26N) and/or HA-BCA3, and the expression was verified by Western blotting (WB) using either anti-c-myc or anti-HA antibody. The cells were lysed 48 h post-transfection and proteins were immunoprecipitated (IP) using anti-c-myc antibody. The precipitates were separated by 15 % SDS-PAGE, blotted and developed with anti-c-myc or anti-HA antibodies. (c) Immunocolocalization of MPMV PR17 and ΔGPDPR13 with BCA3 in HEK-293 cells: the cells transfected with HA-BCA3 and MPMV c-myc-PR17(D26N) or c-myc-ΔGPDPR13(D26N) were stained 48 h post-transfection using tetramethylrhodamine-labelled anti-HA or FITC-labelled anti-c-myc antibodies, and visualized using confocal microscopy.

To confirm these results, we studied co-localization of MPMV PR with BCA3 using confocal microscopy (Fig. 1c). Immunofluorescence staining showed that both c-myc-tagged MPMV PR17 and ΔGPDPR13 co-localized with HA-BCA3. The same pattern was observed when the epitope tags were mutually exchanged, and c-myc-BCA3 and HA-PR17(D26N) were used (data not shown).

Next, we analysed whether BCA3 was incorporated into released MPMV virions. MPMV virions released from HEK-293 cells co-transfected with a MPMV proviral vector encoding active PR (pSARM4) and the vector encoding BCA3 were pelleted through a 20 % sucrose cushion, suspended in PBS, placed on a linear (20–65 %) sucrose gradient and centrifuged to equilibrium in a Beckman SW41 rotor at 35 000 r.p.m. for 16 h. Individual fractions were collected and analysed by Western blotting (Fig. 2a). BCA3 co-migrated with the bulk of MPMV CA, with a peak at a sucrose density of 1.14–1.16 g ml⁻¹ (fractions 5 and 6), which corresponds to the buoyant density of MPMV. Therefore, we constructed a stable cell line expressing BCA3 by using pcDNA4/V5-BCA3 vector that was transfected into HEK-293 cells. Several Zeocin-resistant colonies were isolated and expanded, and production of BCA3 was tested using Western blotting and qPCR. Two clones (labelled D1 and D2, Fig. 2b) were used to ensure the reproducibility of the experiments. Both cell lines were transfected with MPMV proviral DNA, and the expression of BCA3 and MPMV proteins was verified by Western blotting (Fig. 2b, upper panels). At 48 h post-transfection the culture media were filtered through a 0.45 µm filter and collected by centrifugation through a 20 % sucrose cushion in an SW41 rotor at 35 000 r.p.m. for 1 h. Viral pellet proteins were analysed by Western blotting. Although BCA3 expression was significantly lower in this cell line in comparison with transiently transfected cells, BCA3 was identified within the released MPMV particles (Fig. 2b, lower panels).

As exon 3 (residues 75–101) and exon 6 (residues 163–210) of BCA3 display the highest amino acid sequence identity among different species (Table S1), we investigated which of these parts of BCA3 might be responsible for MPMV virion incorporation. To do so, we prepared constructs with a deletion of either exon 3 or exon 6, resulting in expression of Δ3BCA3 or Δ6BCA3, respectively. Virions released from the
cells co-transfected with these constructs and pSARM4 were pelleted as described above, and analysed by Western blot. Both BCA3-derived proteins were expressed at levels similar to that of full-length BCA3 (Fig. 2c). However, whilst Δ3BCA3 was incorporated into MPMV virions similarly to full-length BCA3 (Fig. 2c, lanes 8 and 9), the C-terminally truncated protein Δ6BCA3 was not (Fig. 2c, lanes 5 and 7).

We further analysed the effect of BCA3 on MPMV infectivity in a single-round infectivity assay (Kržová et al., 2012). HEK-293 cells were co-transfected with enhanced-GFP-encoding MPMV proviral vector (pSARM-EGFP) (Newman et al., 2006) and Env-expressing vector (pTMO) (Brody & Hunter, 1992) together with empty or BCA3-encoding vectors. At 48 h post-transfection, the culture supernatants were collected and virion quantification was carried out using quantitative Western blotting followed by ImageQuant TL quantification (Kržová et al., 2012). The number of GFP-positive cells was determined by flow cytometry (BD FACSaria). The mean ± SD percentage of three independent infectivity measurements was calculated.

An additional experiment aimed at investigating the potential role of the GPD in BCA3 incorporation showed that BCA3 is incorporated into MPMV particles that lack the GPD. This was true for GPD deletion constructs with both active (AGPDPR13pSARM4) and inactive (ΔGPDPR13D26NpSARM4) PR (Fig. 3, lanes 5–8). These results confirmed that the GPD is not required for BCA3 packaging into MPMV virions.

In summary, we present BCA3 as a novel MPMV-binding protein that interacts with MPMV PR in HEK-293 cells and is incorporated into MPMV virions. As rhesus BCA3 (Macaca mulatta) shares 96% identity and 98% similarity with its human orthologue (Fig. 1, Table S1), we hypothesize that rhesus BCA3 would also be packaged into MPMV. Our experimental data suggest that the incorporation of BCA3 into MPMV particles is specific because: (i) for BCA3 packaging into MPMV particles, the C-terminal portion of BCA3 (aa 163–210) is critical, and only full-length but not this C-terminally truncated form Δ6BCA3 is incorporated; and (ii) the BCA3 incorporation was not caused primarily by its overexpression, as BCA3 was identified also in MPMV particles released from the low-level BCA3-expressing cell lines. In vitro analysis of the BCA3–MPMV PR interaction would be the final proof of their mutual interaction; however, despite our extensive efforts, we failed to prepare soluble BCA3 in amounts and quality suitable for any in vitro binding experiments, mainly due to its insolubility. Therefore, we were not able to prove whether the BCA3–PR17 interaction is direct or mediated by some other cellular protein.
In three independent experiments, we observed an elevation of infectivity by ~15–20% in the cells infected by MPMV containing incorporated BCA3 in comparison with MPMV released from the cells non-transfected with BCA3 vector or transfected with empty vector. As the biological function of BCA3 is understood poorly, we can only hypothesize that this infectivity increase relates to the published fact that BCA3 translocates from the cytoplasm to the nucleus, where it fulfills several functions in the regulation NFκB-dependent transcription through BCA3’s ability to bind to its p65 subunit and the cyclin D1 promoter in a neddylation- or PKA-phosphorylation-dependent manner (Gao et al., 2006, 2008, 2010; King et al., 2011; Sastri et al., 2005, 2013). BCA3 also encodes a nuclear localization signal at its N-terminal part (R14R15) (Sastri et al., 2005). We might therefore speculate that BCA3 either facilitates nuclear transport of MPMV preintegration complex or facilitates LTR-regulated transcription.

Extensive precise mapping of the MPMV–BCA3 binding site(s) together with determination of the impact of BCA3 on MPMV transcription would be required to understand the role of BCA3 in MPMV biology.

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**Fig. 3.** Role of MPMV PR and its GPD. HEK-293 cells were transfected with the indicated vectors. The culture media were filtered through a 0.45 μm filter 48 h post-transfection and collected by centrifugation through a 20% sucrose cushion in a SW41 rotor at 35 000 r.p.m. for 1 h. Both cellular and virus-associated proteins were analysed by Western blotting using rabbit antibody against MPMV CA (RbxMPMV CA), mouse mAb against HA (MxHA), and rabbit antibodies against MPMV PR (RbxMPMV PR17) and GPD (RbxMPMV GPD).
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References


