Cellular Signalling 27 (2015) 1763–1771

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

The BARD1 BRCT domain contributes to p53 binding, cytoplasmic and mitochondrial localization, and apoptotic function

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ARTICLE INFO

Article history:
Received 7 May 2015
Accepted 15 May 2015
Available online 3 June 2015

Keywords:
BARD1
p53
Mitochondria
Apoptosis
BCL-2

ABSTRACT

BARD1 is a breast cancer tumor suppressor with multiple domains and functions. BARD1 comprises a tandem BRCT domain at the C-terminus, and this sequence has been reported to target BARD1 to distinct subcellular locations such as nuclear DNA breakage sites and the centrosome through binding to regulatory proteins such as HP1 and OLA1, respectively. We now identify the BRCT domain as a binding site for p53. We first confirmed previous reports that endogenous BARD1 binds to p53 by immunoprecipitation assay, and further show that BARD1/p53 complexes locate at mitochondria suggesting a cellular location for p53 regulation of BARD1 apoptotic activity. We used a proximity ligation assay to map three distinct p53 binding sequences in human BARD1, ranging from weak (425–525) and modest (525–567) to strong (551–777 comprising BRCT domains). Deletion of the BRCT sequence caused major defects in the ability of BARD1 to (1) bind p53, (2) localize to the cytoplasm and mitochondria, and (3) induce Bax oligomerization and apoptosis. Our data suggest that BARD1 can move to mitochondria independent of p53, but subsequently associates with p53 to induce Bax clustering in part by decreasing mitochondrial Bcl-2 levels. We therefore identify a role for the BRCT domain in stimulating BARD1 nuclear export and mitochondrial localization, and in assembling mitochondrial BARD1/p53 complexes to regulate specific activities such as apoptotic function.

1. Introduction

The BRCA1-associated RING domain 1 (BARD1) protein is a putative tumor suppressor and a key binding partner of the breast and ovarian cancer susceptibility protein, BRCA1 [1,2]. The BARD1 gene is mutated in a subset of breast and ovarian cancer patients [3–5], and was first discovered in a genetic screen for BRCA1-interacting proteins [6]. BARD1 was later confirmed as the major *in vivo* partner for BRCA1 [1], and the two proteins interact through their N-terminal tail sequences [7]. The formation of a stable BRCA1/BARD1 dimer has a positive influence on BARD1 stability [8], nuclear localization [9] and DNA repair function [10,11]. Furthermore, the BRCA1/BARD1 dimer acts as an E3 ubiquitin (Ub) ligase whose enzymatic activity is impaired by cancer associated gene mutations in the BRCA1 RING domain [12,13]. BRCA1/BARD1 Ub ligase activity is thought to contribute to the regulation of DNA repair [11], mitotic cell division [14] and centrosome duplication [15,16]. The BRCA1/BARD1 heterodimer is also important for cell viability, and loss of either BRCA1 or BARD1 in mice leads to chromosomal abnormalities and early embryonic death due to severe cell proliferation defects [11,17].

Human BARD1 comprises 777 amino acids and, like BRCA1, has an N-terminal RING finger domain and two C-terminal BRCT domains [18]. Unlike BRCA1, BARD1 contains a centrally located ankyrin repeat domain known to mediate protein–protein interactions [19]; the role of this domain in BARD1 is still under investigation although it was reported to contribute to association with p53 in mouse [20]. BARD1 can shuttle between the nucleus and cytoplasm [21,22], and it was previously proposed that while nuclear localization of BARD1 correlated with its role in DNA repair and cell survival [21,23], the cytoplasmic accumulation of BARD1 at mitochondria correlated with induction of apoptosis [24].

BARD1 is transcriptionally up-regulated in response to genotoxic stress in rodent cells [25]. Furthermore, the overexpression of exogenous BARD1 leads to apoptosis and this is partly dependent on

Abbreviations: BRCA1, breast cancer regulatory protein-1; BARD1, BRCA1-associated RING domain 1; CMX-Ros, chloromethyl-X-rosamine; FCS, fetal calf serum; mtHSP70, mitochondrial heat shock protein 70; NES, nuclear export sequence(s); NLS, nuclear localization sequence; OMM, outer mitochondrial membrane; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; YFP, yellow fluorescence protein.

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http://dx.doi.org/10.1016/j.cellsig.2015.05.011
0898-6568/

functional p53, but is independent of and inhibited by BRCA1 as demonstrated in p53 or BRCA1-deficient cell lines [25,26]. We previously discovered a pool of BARD1 at mitochondria whose expression correlated with an increase in Bax oligomerization, mitochondrial membrane permeability and apoptosis [24]. Given that BARD1 apoptotic activity is p53-dependent, and that p53 itself is known to translocate to mitochondria and induce apoptosis [27], we hypothesized that p53 might regulate BARD1 apoptotic function by either (i) recruiting it to mitochondria, (ii) binding BARD1 at mitochondria and/or (iii) modulating its ability to stimulate Bax oligomerization. In this study we identify a key role for the BRCT domain in p53 binding, recruitment to the cytoplasm/mitochondria and apoptotic function. These data reveal that while p53 is not required for targeting of BARD1 to mitochondria, it does form complexes with BARD1 at this organelle and stimulates its role in Bax oligomerization.

2. Materials and methods

2.1. Cell culture and transfections

Human MCF-7 breast cancer cells were grown under standard tissue culture conditions in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). The cell line was mycoplasma-negative. Transient transfections were performed using Lipofectamine2000 (Life Technologies, Vic, Australia) according to manufacturer instructions. Briefly, at 24 h after seeding, cells were transfected at 50% confluence with 2 μg of DNA (per well in a 6-well plate) or 4 μg DNA (for a T25 flask for Western blots). At 6 h post-transfection, the transfection mix was replaced with medium containing 10% FBS. Cells were fixed and processed 24 h post-transfection for fluorescence microscopy.

2.2. Plasmid DNA constructs

The human BARD1 coding sequence was originally provided by Prof. Richard Baer (Columbia University, NY). Most of the pYFP–BARD1 plasmids have been described previously [21,24]. In general, pYFP–BARD1 plasmids were generated by PCR-amplifying the YFP cDNA from the pEYFP-C1 vector (Life Technologies) and inserting it into the NotI site of the respective pFLAG–BARD1 plasmids. pYFP–BARD1 (551–777) was described previously [24]. New pYFP–BARD1 plasmids expressing different BARD1 sequences were constructed by amplifying BARD1 cDNA sequences and inserting them into the indicated restriction sites of pEYFP-Cl: (BARD1 425–525, Xho I/Sal I; 525–567, Sac I/Xma I). The forward and reverse primer sequences used are outlined in the Supplementary Methods section. The BARD1 internal deletion constructs BARD1 Δ425–525 and Δ525–567 were constructed by PCR-amplifying the flanking sequences of each deletion and re-ligating and inserting them into pEYFP-C1. Full details of the primer sequences and restriction sites are in the Supplementary Methods. Plasmid sequences were checked by DNA sequencing.

2.3. Isolation of mitochondrial extracts

Mitochondrial fractions were enriched from cells using the Qproteome mitochondrial isolation kit (Qiagen). Cells were lysed to isolate cytosolic proteins. Plasma membranes and organelles such as nuclei, mitochondria and endoplasmic reticulum were pelleted by centrifugation at 1000 ×*g* for 10 min. The pellet was then resuspended in Disruption Buffer, using a narrow-gauge needle and re-centrifuged to pellet nuclei and cell debris. The supernatant was again centrifuged at 6000 ×*g* for 10 min to pellet the mitochondria, which were snap frozen in liquid nitrogen, thawed on ice and analyzed by immunoprecipitation and/or western blot analysis.

2.4. Immunoprecipitation of mitochondrial proteins

(a) *BARD1 pull-down*. 50 μl of Dynalbeads (Life Technologies) was used per immunoprecipitation. Beads were washed in 0.1 M phosphate buffer pH 7.4 for 2 min at room temperature. Washed beads were then resuspended in 0.1 M PBS pH 7.4 and incubated with 1 μg IgG or BARD1 antibody overnight at 37 °C, beads were washed with PBS with 0.1% (*w*/*v*) BSA, followed by a wash with 0.2 M Tris pH 8.5 for 4 h at 37 °C, and PBS/Triton X-100 for 10 min. Beads were resuspended in 50 mM Tris pH 7.5, 150 mM NaCl, 0.05% NP-40 (NET2) buffer and then incubated with 2 mg total or mitochondria-enriched lysate, washed and analyzed by SDS-PAGE and immunoblotting.

(b) *p53 pull-down*. For IP of p53 we used Protein A-Sepharose beads (GE Healthcare Bio-Sciences) equilibrated in lysis buffer (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.5% NP-40). Beads (30 μl/reaction) were pre-coated with 3 μg rabbit p53 antibody (sc-6243, Santa Cruz Biotech, CA) or rabbit IgG (Sigma, MO) for 1.5 h at 4 °C and washed with lysis buffer three times. Cells were lysed in lysis buffer for 30 min on ice, then centrifuged 15,000 ×*g* for 10 min at 4 °C. 100 μg extract was removed, denatured in 2× Laemmli buffer and run as an input sample. 1 mg of the supernatant (total protein lysate) was incubated with beads for 1.5 h at 4 °C and immunocomplexes pelleted, washed in RIPA buffer for 5 min before centrifugation, then denatured and analyzed by immunoblotting. The following antibodies were used for detection: GFP monoclonal (11814460001, Roche, 1:1000); BARD1 rabbit polyclonal (A300-263A, Bethyl Laboratories, 1:1000), 53BP1 monoclonal (PC712, Oncogene, 1:1000) and p53 monoclonal (sc-126, Santa Cruz Biotec, 1:1000).

2.5. Immunoblotting

Cell lysates were denatured in 100 mM Tris–HCl (pH 6.8), 20% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol, 5% SDS then separated by SDS-PAGE and analyzed by Western blot. Approximately 80 μg of protein extract was loaded per lane, resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore Corporation). The membranes were blocked with 5% skim milk powder in PBST (PBS containing 0.2% Tween 20) for 1 h followed by incubation with primary antibody for 2 h, using antibodies against BARD1 (A300-263A, Bethyl Laboratories, 1:1000), PCNA (610665, BR Transduction Laboratories, 1:5000), β-tubulin (T6074, Sigma, 1:3000), mtHSP70 (MA3-028, Affinity Bioreagents, 1:300) and p53 (sc-6243, Santa Cruz Biotec, 1:500). Blots were incubated with secondary horseradish peroxidase-conjugated antibodies (1:10,000, Sigma) for 1 h followed by detection using enhanced chemiluminescence (ECL; Amersham Biosciences). For detection of ectopic YFP–BARD1 proteins, cells were transfected with YFP–BARD1 plasmid and processed for mitochondrial extraction 24 h later.

2.6. Immunofluorescence microscopy

Cells were grown on coverslips at 60% density and fixed in 3.7% formalin/PBS for 20 min, followed by permeabilization with 0.2% Triton X-100/PBS for 10 min at 24 h post-transfection, and incubated with various antibodies as described [24]. For analysis of mitochondrial membrane permeability, live cells were incubated with 100 nM MitoTracker CMX-Ros (Molecular probes) in medium for 30 min at 37 °C, then washed and fixed in ice-cold acetone:methanol (1:1) for 3 min at room temperature. For antibody staining of fixed cells, cells were blocked with 3% bovine serum albumin in PBS for 1 h, followed by addition of primary antibodies as follows: rabbit polyclonal Ab against BARD1 (A300-263A, Bethyl Laboratories, 1:500); rabbit polyclonal