Biochimica et Biophysica Acta 1810 (2011) 432–438

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Cell cycle-dependent conjugation of endogenous BRCA1 protein with SUMO-2/3

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ARTICLE INFO

Article history:
Received 7 May 2009
Received in revised form 21 October 2010
Accepted 7 December 2010
Available online 13 December 2010

Keywords:
BRCA1
SUMO-2/3
Cell cycle
Protein processing
Post-translational
Oxidative stress

ABSTRACT

Background: BRCA1, the main breast and ovarian cancer susceptibility gene, has a key role in maintenance of genome stability, cell cycle and transcription regulation. Interestingly, some of the numerous proteins which interact with BRCA1 protein undergo conjugation with small ubiquitin-like modifiers (SUMO). This post-translational modification is related to transcription, DNA repair, nuclear transport, signal transduction, and to cell cycle stress response.

Methods and results: Protein sequence analysis suggests that sumoylation target sites belong to the RING finger and BRCT domains (BRCA1 C-terminus), two crucial regions for BRCA1 function. Moreover putative SUMO interacting motifs are present in the sequence of many proteins of BRCA1 network. Using immunoprecipitations and western blotting, we show the conjugation of endogenous nuclear BRCA1 protein with SUMO-2/3. BRCA1 conjugation with SUMO-2/3 is linked to the cell cycle in a cell line dependent manner since no cell cycle dependence of sumoylation is observed in MCF7 breast cancer cells. In contrast, BRCA1 conjugation with SUMO-2/3 is linked to the oxidative stress independently to the cell line, in DU145, MCF7 and 293 T cells.

Conclusion and general significance: Our data reveal a new BRCA1 regulation pathway implying sumoylation in response to cell cycle progression and oxidative stress, providing a possible mechanism for the involvement of BRCA1 gene in tumorigenesis.

1. Introduction

BRCA1 is one of the main breast and ovarian cancer susceptibility genes [1]. It has a crucial role in several cell processes including cell cycle progression and apoptosis, maintaining genomic integrity and chromosome structure (for a review see [2,3]). Depending on the cell cycle, BRCA1 protein shuttles from cytoplasm to nucleus [4] or is exported towards the cytoplasm [5,6]. BRCA1 protein is a scaffold for large supramolecular complexes, interacting with numerous proteins involved in transcription, DNA repair and cell cycle control. Interestingly, many of these are conjugated to small ubiquitin-like modifiers [7] e.g. p53 [8] or RB [9].

SUMO posttranslational modifications are involved in several cellular functions, notably DNA repair or cell cycle regulation [10]. Many transcription factors are sumoylated and some sumoylation events are dependent on a nuclear localisation signal [11]. Four SUMO paralogues have been described in mammals. SUMO-1 would modify proteins homogeneously distributed in the cytoplasm. SUMO-2 and SUMO-3 have a very high level of similarity and SUMO-2/3 conjugation is implicated in cellular stress response [12]. It is noticeable that SUMO modification also has a role in protein transport: although SUMO-1 regulates cytoplasm-nucleus traffic, SUMO-3 would regulate intranuclear localization [13].

These features of sumoylation mechanisms and of BRCA1 function, together with the observation that sumoylation targets often belong to the same protein complex or pathway [11] lead us to test the hypothesis of BRCA1 sumoylation by SUMO-2/3. Our data show that nuclear BRCA1 protein undergoes conjugation by SUMO-2/3 in relationship with cell cycle and oxidative stress, depending on the cell line.

2. Materials and methods

2.1. Cell culture

MRC5, MCF7, DU145 and 293 T cell lines were grown in DMEM containing 10% foetal calf serum, at 37 °C, 5% CO_2 and 90% humidity.

Abbreviations: SUMO, small ubiquitin-like modifiers; SIM, SUMO interacting motifs; BRCT, BRCA1 C-terminus

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doi:10.1016/j.bbagen.2010.12.001

2.2. Transfections

293 T cells were transfected with ExGen 500 (Fermentas Life Sciences, Saint-Rémy-lès-Chevreuse, France) for 24 hours using 4 μg of a single plasmid or 2 μg of each plasmid when two plasmids were transfected simultaneously. Plasmids pcDNA3-His_6-SUMO-2 and Fl4-BRCA1-wt/pCMV were kind gifts of Dr. A Vertegaal [14] and Dr. R Baer [15] respectively.

2.3. Nuclear extracts and whole cell lysates

Nuclear fractions were prepared as described [16]. Briefly, cells were lysed in lysis buffer (20 mM HEPES pH 7.95, 20 mM NaCl, 0.5% NP40, 1 mM DTT, 100 mM Na_3VO_4) supplemented with Complete™ Mini protease inhibitor mixture (Roche Molecular Biochemicals, Meylan, France) and 20 mM NEM on ice for 5 minutes. After centrifugation, the cytoplasmic supernatant was removed and the nuclear proteins were extracted from the pelleted nuclei by incubation in 20 mM HEPES pH 7.95, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% (vol/vol) glycerol, 0.2 mM EDTA, 1 mM DTT, Complete™ Mini protease inhibitor mixture and 20 mM NEM on ice for 30 minutes. After centrifugation, the protein concentration of the supernatant was determined using a BCA protein assay (Pierce, Montluçon, France).

Whole cell lysate were prepared according to Jaffray and Hay [17] in protein denaturing buffer (2% SDS, 50 mM Tris–HCl, pH 7.5, 10 mM iodoacetamide, 100 mM Na_3VO_4 and protease inhibitors) followed by dilution 20 times in renaturation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 mM β-mercaptoethanol, 100 mM, Na_3VO_4 and protease inhibitors). Nuclear extracts were resuspended (vol/vol) in protein denaturing buffer and diluted in renaturation buffer as well.

2.4. Cell synchronisation

Cells were grown to 75% confluence then starved in DMEM containing 1% foetal calf serum for 48 hours and finally released in completed medium containing 10% foetal calf serum.

2.5. Immunoprecipitation and western blot analysis

Equal amounts of nuclear proteins were immunoprecipitated by 2 μg of antibodies in PBS supplemented with 0.1 M NaCl, 100 mM Na_3VO_4, 0.2 mM EDTA, Complete™ Mini protease inhibitor mixture (Roche Molecular Biochemicals) and 20 mM NEM, for 1 hour on ice. The antibodies used were as follows: Ab-1 anti-BRCA1 antibodies (Calbiochem, Lyon, France), C20 anti-BRCA1 antibodies (Santa Cruz Biotechnology, Santa-Cruz-CA, USA), anti-Flag antibodies (Sigma, Saint Quentin Fallavier, France) and mouse or rabbit IgG as irrelevant antibodies for control. Immune complexes were collected on protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz-CA, USA) for 30 minutes at 4 °C, washed three times in ice-cold PBS. For C20 immunoprecipitation, washes were as follows: once in PBS plus 0.2 M NaCl, three times PBS plus 0.1 M NaCl, then once in PBS.

Samples were electrophoresed on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Millipore, Saint-Quentin, France), and blocked in PBS containing 5% skimmed milk and 0.05% Tween. Blots were incubated with primary antibodies as follows: Ab-1 anti-BRCA1 diluted 1:60, anti-GMP-2 antibodies directed against SUMO-2/3 (Zymed, San Francisco-CA, USA) diluted 1:2500, C20 anti-BRCA1 diluted 1:250, C4 anti-actin (MP Biomedicals France, Illkirch, France) diluted 1:50 000 and anti-HIS diluted 1:5000, (Clontech, Saint Quentin Yvelines, France). Secondary antibodies were peroxydase-conjugated anti-rabbit or anti-mouse immunoglobulins (Santa Cruz Biotechnology) diluted 1:5000 in blocking solution. Proteins were detected by enhanced chemiluminescence (ECL) detection system Amersham ECL (GE Healthcare Life Sciences, Saclay, France).

Table 1
Detection of human BRCA1 potential SUMO conjugation sites.

	Position	Sequence	Score
1	K1160	LDDGE I*K*ED TSFAE	0.94
2	K119	NSPEH L*K*DE VSIIQ	0.91
3	K32	ICLEL I*K*EP VSTKC	0.84
4	K1601	SSTSA L*K*VP QLKVA	0.80
5	K1690	TTHVV M*K*TD AEFVC	0.80
6	K795	STLGK A*K*TE PNKCV	0.79
7	K109	NSYNF A*K*KE NNSPE	0.79
8	K947	NAKCS I*K*GG SRFCL	0.77
9	K830	NDTEG F*K*YP LGHEV	0.74
10	K912	KEENQ G*K*NE SNIKP	0.67

Consensus-like sumoylation sites were selected using SUMOplot analysis tool (http://www.abgent.com/doc/sumoplot). Only sites with high probability, i.e. a score higher than 0.60, are displayed.

3. Results

3.1. Protein sequence analyses

First analysis of human BRCA1 protein sequence analysis by the SUMOplot™ software displays several possible sites for sumoylation, ten of which have a probability score higher than 0.60 and five a score equal or higher than 0.80 (Table 1). Despite the high level of conservation among primates, sequence comparison by CLUSTAL W multiple sequence alignment showed that two of the consensus-like sumoylation sites, surrounding K32 and K1690, are conserved among species (supplemental data, Figs. 1 and 2). BRCA1 N-terminus which harbours the RING finger is very highly conserved so that the first potential site around K32 is common to all considered species. The second site including K1690 in human is located in the first BRCT repeat; despite sequence variation, its probability of sumoylation remains high in all species (Table 2). Thus, both sites are likely candidates for *in vivo* modification.

Some of the proteins belonging to BRCA1 network are sumoylated (supplemental Table 1) and SUMOplot™ analysis might suggest sumoylation of a larger number of BRCA1 partners. Moreover some of these proteins such as Rad51 or ACACA and BRCA1 itself also display one or several putative SUMO interacting motifs (SIM) [18] (supplemental Table 2). This observation would imply a role of SUMO conjugation in the interaction between BRCA1 protein and its partners.

3.2. Immunoprecipitation of endogenous nuclear BRCA1 protein conjugated to SUMO-2/3

Since BRCA1 is a mainly nuclear protein [2–4], nuclear lysates from DU145 prostate cancer cells and MRC5 human embryo lung cell line were immunoprecipitated with anti-BRCA1 antibodies followed by immunoblotting with anti-SUMO-2/3 antibodies (Fig. 1A). The results demonstrated that nuclear endogenous BRCA1 protein is covalently conjugated to SUMO-2/3, which is compatible with the molecular

Table 2
The potential SUMO conjugation site in the first BRCT domain of BRCA1 protein is conserved among species.

Species	Position	Sequence	Score
Human	1690	THVV **MKTD** AEFV	0.80
Monkeys	1690	THVV **MK̄TD** AEFV	0.80
Dog	1693	THVI **MK̄TD** AEFV	0.80
Bovin	1683	THVI **MK̄TD** PEFV	0.80
Mouse	1633	THVI **IKTD** AEFV	0.94
Rat	1636	THVI **IK̄TD** AEFV	0.94
Chicken	1581	THVI **MK̄TD** EELV	0.80
Xenopus	1414	THVI **MK̄TD** AELV	0.80

Consensus-like sumoylation sites were selected using SUMOplot analysis tool (http://www.abgent.com/doc/sumoplot). Only sites with high probability, i.e. a score higher than 0.60, are displayed.