Identification of an Apoptotic Cleavage Product of BARD1 as an Autoantigen: A Potential Factor in the Antitumoral Response Mediated by Apoptotic Bodies

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ABSTRACT

We have shown previously that rats can be cured from induced peritoneal colon carcinomatosis by injections of apoptotic bodies derived from tumor cells and interleukin 2. This curative treatment generated a tumor-specific cytotoxic T-cell response associated with a humoral response. Autoantibodies from sera of cured rats strongly recognized a M, 67,000 protein from apoptotic bodies and weakly reacted with a protein of M, 97,000 in PROb parental cells. We now show that these autoantibodies are directed against BARD1, originally identified as a protein interacting with the product of the breast cancer gene 1, BRCA1. We demonstrate that the M, 67,000 antigen is a cleaved form of BARD1 present in apoptotic bodies derived from rat and human colon and mammary carcinoma cell lines. Moreover, we show that the cleavage site of BARD1 is located NH2 terminally but downstream of the RING domain essential for BARD1 and BRCA1 protein interaction. In vitro studies using [35S]methionine-labeled human BARD1 and apoptotic cellular extracts derived from SW48 carcinoma cells indicate that BARD1 proteolysis occurs at an early stage of apoptosis and in a cell cycle-dependent manner. This hydrolysis is inhibited by EGTA, and the calpain inhibitor I, N-acetyl-leu-leu-norleucinal, but not by several caspases inhibitors, suggesting that BARD1 is hydrolyzed by the calcium-dependent cysteine proteases, calpains. Thus, the highly immunogenic form of cleaved BARD1 could contribute to the antitumoral response mediated by apoptotic bodies.

INTRODUCTION

Colon cancer is the second most common fatal malignancy in the Western world (1). Because of their resistance to chemotherapeutic treatments, human colorectal cancers often produce refractory metastases and recurrences (2). Advances in the field of cancer immunotherapy have provided vaccines for the treatment of minimal residual disease after surgery. Thus, to induce specific immune recognition of the tumor-associated antigens, recent vaccine strategies have used tumor-derived cells for the treatment of human colon cancer (3) or cellular materials containing cryptic or tumor-associated antigens for the treatment of melanoma (4, 5) and B-cell lymphoma (6).

Our laboratory has developed a strategy for the treatment of rats with induced peritoneal colon carcinomatosis, aimed at increasing the immunogenicity of colon cancer cells and providing greater stimulation of the immune system. Combined i.p. injection of IL-2 and the differentiation agent NaB cured rats with peritoneal carcinomatosis by injections of apoptotic bodies derived from PROb parental cells (13). This curative treatment generated a tumor-specific cytotoxic T-cell response (14) and the production of autoantibodies that strongly recognized a M, 67,000 antigen specifically expressed in apoptotic bodies and weakly reacted with a M, 97,000 protein present in parental PROb cells (13).

In this study, we demonstrated that the M, 67,000 antigen is a cleaved form of the M, 97,000 protein generated during apoptosis. We report the immune-screening of a PROb cDNA library with sera from cured rats that led to the identification of the M, 67,000 protein as a proteolytic fragment of BARD1, a protein interacting with the tumor suppressor gene product BRCA1 (15), and the mechanism of the cleavage of BARD1 during apoptosis.

MATERIALS AND METHODS

Cell Culture. PROb rat colon adenocarcinoma (obtained from ECACC, Salisbury, United Kingdom) and REGb rat colon carcinoma cells (a gift from Dr. F. Martin, INSERM, Dijon, France) were both derived from a dimethyl-hydrazine-induced cell line (16). The rat mammary carcinoma 13762, the human colon carcinoma SW48, and mammary carcinoma MCF7 were obtained from ECACC. Cells were grown in monolayer cultures at 37°C in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS and 2 mM glutamine. Cells were passaged with 0.025% trypsin and 0.02% EDTA and routinely tested for Mycoplasma contamination with Hoechst labeling.

Immunological Screening and Cloning of the Rat BARD1 cDNA. A cDNA library of the PROb rat carcinoma cell line was constructed in the expression vector ATriplEx (Clontech, Palo Alto, CA). One million of plaques were screened with sera from cured rats after vaccination with apoptotic bodies/IL-2 (13). Antibodies against Escherichia coli were removed from rat antiserum by incubating sonicated E. coli with serum diluted to one-tenth in PBS + 5% nonfat milk dry for 4 h at room temperature and then centrifuged at 13,000 × g for 10 min. The insert of 456 bp (F1 fragment) was sequenced, and the sequence was submitted to the National Center for Biotechnology Information (NCBI) gene bank analysis. It presented a strong homology with the human protein BARD1. Cloning of the complete cDNA of rat BARD1 was achieved using the PROb cDNA library constructed in SMART PCR kit (Clontech). Internal primers for the rapid amplification of cDNA ends PCR were chosen from the cloned insert as recommended by the manufacturer.

Cloning of the Human BARD1 cDNA. Three fragments of human BARD1 cDNA were amplified from total RNA extracted from SW48 human colon carcinomatosis cell line. Fragments A, B, and C were obtained using the following primers: fragment A: sense primer, R1355 antisense primer, B202N (17); fragment B: sense primer, B202A (17) antisense primer, 5’-CACCAAT-GCCCTATGCTGGAGC-3’; fragment C: sense primer, 5’-GAAGTAGT-
GACTCCCTGAAAGG-3’/antisense primer, 5’-TCAGCTGTCAAGAGGAA-GCAACCTC-3’. Each fragment was cloned into the pGEM plasmid (Promega Corp., Madison, WI) and then excised using NotI-PstI/PstI-HindII/HindIII/ BstXI, respectively, purified, and then ligated together into the NotI/BsXI sites of pGEM.

Apoptosis Induction and Purification of Apoptotic Bodies. Apoptosis was induced by NaB treatment. Cells at different stages of confluence were treated in complete medium at 37°C with 5 mM NaB (Sigma, St. Quentin Fallavier, France) for different time periods as indicated in the figure legends. Apoptotic bodies were purified as described previously (18).

 Production and Purification of Rat BARD1 F1 Fragment. The F1 fragment of the rat BARD1 was excised from the plasmid (ATrIplex) derived from the cDNA library and inserted in-frame into the p Berlin site of pQE32 plasmid (Qiagen, Courtaboeuf, France). The resulting fusion protein, containing a 6His tag placed at the NH2 terminus of the BARD1 F1 fragment, was expressed in E. coli and then purified by affinity chromatography on Ni-NTA resin using the manufacturer’s recommendations for the QIAexpressionist kit (Qiagen).

Mouse Immunization and Monoclonal Antibody Production. Balb-c mice (Iffa-Credo, l’Arbresle, France) received s.c. injections of 100 μg of rat BARD1 F1 fragment in 0.1 ml of Freund’s incomplete adjuvant (Life Technologies, Inc.) emulsified in 0.1 ml of sterile PBS. 0.5% Triton X-100, in intervals of 3 weeks. Splenocytes from one mouse were fused with mouse SP2 myeloma (ECACC) in the presence of polyethylene glycol 1500 (Boehringer Mannheim, Meylan, France). Hybridomas were plated in 96-well plates in complete medium supplemented with 20% FCS, hypoxanthine-aminopterin-thymidine (Sigma), and 1.5 mg/ml of recombinant IL6 (RD Systems, Minneapolis, MN). Hybridoma supernatants were tested by ELISA using purified BARD1 F1 fragment as antigen.

Immunoprecipitation. Apoptotic bodies were extracted on ice with 2% Triton X-100 in PBS supplemented with an EDTA-free protease inhibitor cocktail (Boehringer Mannheim) for 30 min. The extract was centrifuged for 15 min at 13,000 × g, and the supernatant was incubated with rabbit polyclonal antibodies directed against human BARD1 (669D; Refs. 15 and 19) diluted to 1:1000. After 4 h incubation, with constant end-over-end mixing, immune complexes were immunoprecipitated by adding 50 μl of antirabbit IgG agarose. The agarose-bound immune complexes were washed with 1% Triton X-100 in PBS containing protease inhibitors and extracted from the agarose beads by boiling in reducing sample buffer for electrophoresis and immunoblotting as described below.

Western Blots. Electrophoresis was conducted under denaturing conditions (SDS-PAGE; Ref. 20). The proteins were transferred to a polyvinylidene difluoride 0.45 μm filter (Millipore, Bedford, MA) and blotted with primary antibodies. Secondary antibodies were conjugated to horseradish peroxidase and used diluted to 1:15,000 (Sigma). The immune complexes were visualized by chemiluminescence using Super Signal kit (Pierce, Rockford, IL).

 Coupled in Vitro Transcription/Translation and in Vitro Protein Cleavage Assay. [35S]Methionine-labeled human BARD1 was in vitro transcribed and translated using the TNT coupled reticulocyte lysate systems kit (Promega). One μg of plasmid was used in a 50-μl transcription/translation reaction containing 4 μl of translation grade [35S]methionine (DuPont NEN, Le Blanc Mesnil, France). For the in vitro cleavage, 2 μl of the transcription/translation product were incubated with apoptotic or nonapoptotic cellular extracts prepared in DIV buffer [20 mM HEPES (pH 7.5), 10 mM NaCl, 1.5 mM MgCl2, 0.1% SB14, and 0.5 mM phenylmethylsulfonyl fluoride] at 37°C during the indicated period of time. Hydrolysis products were then separated by SDS-PAGE and revealed by autoradiography using Phosphorlmager 4455i (Molecular Dynamics, Sunnyvale, CA). The cleavage inhibition was evaluated by adding caspases inhibitors or proteasome inhibitor (lactacystin; Calbiochem, Reutlingen, Germany) as described above.

Cell Cycle Synchonization. SW48 cells were arrested in G0 by contact inhibition in 175-cm2 flasks. After 3 days of confluence, the cells were split 1:10 in 75-cm2 flask at a concentration of 3 × 105 cells/flask. Twelve, 20, 28, 36, and 44 h after seeding, cells were treated with 5 μm NaB during 24 h and harvested. To determine the cell cycle distribution at each time point, the contents of each flask were trypsinized, washed three times in 10 ml of ice-cold PBS, and fixed with 1 ml of ice-cold 70% ethanol added dropwise for 16 h at −20°C. The fixed cells were pelleted, resuspended in 500 μl of PC buffer [96% 0.2 m NaHPO4, 4% 0.1% m citric acid (pH 7.8)] and left 30 min at room temperature. Cells were then washed and resuspended in 500 μl of propidium iodide (50 μg/ml) in staining solution (PBS, 0.12% Triton X-100, 0.12 mM EDTA, and 100 μg/ml RNase A), incubated for 30 min at 37°C, and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). For the in vitro protein cleavage assay, cells were scraped, and cellular extracts were prepared as described previously.

Caspase Activity Assay. For caspase activity assays, 10 μg of cellular extracts were diluted in 100 μl of DIV buffer. Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, acetyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin, and acetyl-Ile-Val-Thr-Asp-7-amino-4-methylcoumarin (Bachem, Bubendorf, Switzerland) substrates for caspase 3, 6, and 8, respectively, were added at a final concentration of 50 μM. Cleavage activity was monitored on Fluorolite 1000 (Dynatech Laboratories).

Cell Fractionation. Cells were cultured in a 75-cm2 flask, trypsinized, and resuspended in 100 μl of CEB buffer [50 mM HEPES (pH 7.4), 50 mM KCl, 2 mM MgCl2, 1 mM DTT, and 10 μM cytochalasin B]. The resuspended cells were left on ice for 30 min and then homogenized by 50 strokes in an ice-cold Dounce homogenizer. Nuclear fraction was prepared by centrifugation at 800 × g for 10 min at 4°C. The pellet was resuspended in CEB buffer and stored at −80°C. Mitochondrial and postmitochondrial fractions were obtained after centrifugation at 13,000 × g for 10 min at 4°C. Both the mitochondrial pellet resuspended in CEB and the postmitochondrial fraction were aliquoted and stored at −80°C until required.

RESULTS

Cloning of the cDNA Encoding the p67 kDa Protein. The immunoscreening of the cDNA ATriplex bank with rat sera cured of carcinoma by treatment with apoptotic bodies/IL-2 led to the identification of a positive insert of 456 bp of the rat BARD1 gene (fragment F1). This fragment is spanning the ankyrin repeats and the BRCT domain encoding amino acids 460 through 611 (Fig. 1). The complete rat BARD1 cDNA revealed an open reading frame coding for 768 amino acids, slightly longer than mouse (765 amino acids) but shorter than the human sequence (777 amino acids; Fig. 1). Table 1 shows that the rat and mouse orthologs of BARD1 share 88% identity, whereas rat and human proteins share 65% identity. This last value is similar to that observed for mouse and human BARD1 (67%). Higher levels of conservation are found within the conserved domains (RING finger, ankyrin repeats, and BRCT domain) of BARD1, 94–95% between rat and mouse BARD1. This percentage was lower between rat and human or mouse and human proteins, 91% within the ankyrin repeats, 86% within the RING finger, and 80% within the BRCT domain.

The p67 kDa Protein Is a Fragment of BARD1. To prove the identity of BARD1 as the p67 kDa protein, we determined its expression in tumor cells and apoptotic bodies. For this purpose, we produced monoclonal antibodies, clone 6D10, against the F1 fragment. When tested on rat carcinoma PROb and REGb cells, 6D10 monoclonal antibody recognized a protein of 97,000 molecular weight (Fig. 2) which was considered as BARD1. This result was confirmed by using the 6D10 polyclonal antibody (15, 19) against human BARD1 (data not shown). Treatment of human colon SW48 or mammary MCF7 carcinoma cells with NaB showed a similar result after blotting with the 669D polyclonal antibody (Fig. 2). Finally, the immunoprecipitation of BARD1 from human or rat carcinoma-derived apoptotic bodies with the 669D polyclonal antibody, followed by immunoblotting with cured rat serum, detected a protein of M67,000 (Fig. 2D). Together, these results prove the identity of the p67 kDa protein as BARD1.
BARD1 or the WFS polyclonal antibody directed against the NH₂ terminal of mouse BARD1 (amino acids 101–114; Ref. 21), we observed that the WFS antibody failed to recognize the Mr 67,000 molecule, whereas the 669D antibody recognized the Mr 67,000 molecule in both kinds of apoptotic bodies (Fig. 2C). This strongly suggests that the cleavage site of BARD1 is located NH₂ terminally but downstream of the RING domain (amino acids 40 – 84; Fig. 1) essential for BARD1 and BRCA1 protein interaction (15).

Cell Cycle-dependent Cleavage of BARD1 during Apoptosis. We examined the effect of NaB treatment on BARD1 cleavage in adherent SW48 cells or in the apoptotic bodies recovered from the supernatant. Fig. 3A shows that the lysates of adherent cells completely cleaved the radiolabeled hBARD1 after 4 h of incubation, because the full-length hBARD1 protein completely disappeared and a protein of Mr, 67,000 appeared (Fig. 3A, lines 2). However, the incubation with lysates from apoptotic bodies had no effect on hBARD1 hydrolysis (Fig. 3A, lines 1). These results indicate that proteolytic activity involved in BARD1 cleavage occurred before the ultimate step of apoptosis, which leads to the formation of apoptotic bodies and cell detachment.

The analysis of the kinetics leading to hBARD1 hydrolysis with lysates of SW48 adherent cells treated for 24 h with 5 mM NaB showed that the cleavage is completed within 1 h (Fig. 3B). Further analysis of the kinetic induction of this cleavage by NaB showed that the p67 protein appears after 4 h of NaB cell treatment (Fig. 3C), and the cleavage was nearly complete within 12 h (Fig. 3C).

Interestingly, the hydrolysis of hBARD1 is regulated in a cell cycle-dependent manner and was predominantly observed during the G₀-G₁ state (Fig. 4, top). This was demonstrated by the addition of NaB 32 h after of cell plating, at which point 80% of the cells were in G₀-G₁ and only 6% in G₂-M phase, which led to a complete conversion of hBARD1 to p67 (Fig. 4, bottom).

hBARD1 Cleavage Is Mediated by Calpains. To further define the proteolytic activity responsible for hBARD1 cleavage, different cellular organelle preparations from SW48 cells treated with NaB were tested. It appeared that both nuclear and mitochondrial preparations were able to cleave hBARD1. The 13,000 × g supernatant of the organelle preparation had no effect (Fig. 5). The effector protease cascade of the apoptotic process comprises cysteine proteases such as caspases or calpains. Therefore, we determined the activities of caspases during the treatment of SW48 cells with NaB by using specific substrates, and we found that caspases 3, 6, and 8 activities progressively increased (Fig. 3D), caspase 3 being the most active.

Table 1 Percentage of homology between BARD1 and its consensus domains in human and rodent species

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<th>BRCT</th>
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<td>86.6</td>
<td>90.9</td>
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Fig. 1. Alignment of the amino acid sequences of human, rat, and mouse BARD1. Sequences corresponding to the RING finger, the three ankyrin repeats, and the two tandem BRCT domains are highlighted. The Q564H mutation of human BARD1 is also conserved and indicated. The sequences were aligned by introducing gaps to maximize amino acid sequence identity; the values for amino acid identity were then calculated by considering each gap as a single mismatch (Table 1). The sequence data of rat BARD1 cDNA are available from EMBL/GenBank/DDBJ under accession number AF182946.

Fig. 2. Expression of BARD1 in cancer cells. Western blot analysis of proteins from rat and human carcinomas cells and their apoptotic bodies is revealed with 6D10 antirat BARD1 monoclonal antibody (A) and 669D antihuman polyclonal antibody (B). ab., apoptotic bodies.
after 6 and 12 h of NaB treatment. The use of peptide inhibitors for caspases showed that the benzylxycarbonyl-Val-Ala-Asp-fluoromethylketone, a pan inhibitor of caspases, slightly inhibited hBARD1 hydrolysis at high concentration (100 μM; Fig. 6A). However, specific inhibitors of caspase 3, the benzylxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone or caspase 6, benzylxycarbonyl-Val-Glu-Ile-Asp-fluoromethylketone, had no effect (not shown). This phenomenon was confirmed by the fact that purified caspase 3 had no effect on hBARD1 hydrolysis (Fig. 6B). These results clearly showed that hBARD1 is not a direct substrate for caspases. Moreover, the proteasome inhibitor lactacystin (22) did not block hBARD1 proteolysis, even at concentrations of 100 μM (Fig. 6A), thus excluding that the proteasome could be involved in this mechanism.

A number of proteins that are degraded during apoptosis are targets of calpains. A possible mechanism for the activation of calpains involves the cleavage of the in vivo calpain inhibitor, calpastatin (23). Our results show that: (a) calpastatin was completely cleaved in SW48 cells after 12 h of NaB treatment (Fig. 3E); and (b) the calpain inhibitor I ALLnL and EGTA strongly inhibited hBARD1 hydrolysis in a dose-dependent manner (Fig. 6A). Altogether, these results strongly suggest that hBARD1 was hydrolyzed by calpains.

**DISCUSSION**

The induction of tissue-specific autoimmunity presents a new principle in the therapy of many important cancers (24, 25). In the course of autoimmune diseases, autoantibodies are able to increase the efficiency of antigen capturing by professional antigen-presenting cells (26, 27), leading to the enhancement of the presentation of nondominant pathogenic determinants and T-cell activation (28). It was therefore interesting to investigate the humoral response revealed in the sera of cured rats after treatment by apoptotic bodies/IL-2 (13) and the mechanisms involved.

In this paper, we report that the p67 kDa protein recognized by the sera of rats cured and subsequently immunized against cancer is a proteolytic fragment of BARD1. This identity was confirmed by: (a) the cloning of the rat BARD1 by immunoscreening with the sera of cured rats; (b) the demonstration that the full-length BARD1 (M, 97,000) is expressed in colon and mammary cancer cells, whereas the truncated form (M, 67,000) is expressed in apoptotic bodies; and (c) the immunoprecipitation of the p67 kDa with specific antihuman BARD1 antibodies followed by Western blotting with the sera of cured rats.

BARD1 was reported to play a critical role in BRCA1-mediated tumor suppression. Both proteins possess NH$_2$-terminal RING finger motifs and COOH-terminal BRCT domains, with the former responsible for the BARD1/BRCA1 heterodimer formation (15). The cloning of the rat BARD1 gene confirms the expected high homology (88%) with the mouse molecule (29). However, the homology with the human protein is only 65%, similar to the identity between mouse and human molecules. These comparisons suggest that the primary sequences of BRCA1 and BARD1 have diverged at a similar rate during mammalian evolution, consistent with the notion that both proteins function as components of the same heteromeric complex (29).

BARD1, in association with BRCA1, colocalizes with proliferating...
addition, our results show that calpastatin, an in vivo calpain inhibitor (23), was hydrolyzed by apoptotic SW48 cell lysates, thus activating BARD1 proteolysis by calpains, as it was observed in other systems (40, 41).

Several proteins such as poly(ADP-ribose) polymerase, lamin, U1—70 kDa, DNA-dependent protein kinase (DNA-PKcs), and nuclear mitosis antigen (NuMA) are fragmented during apoptosis and have been reported to become autoantigenic targets in systemic autoimmune diseases (reviewed in Ref. 12). Our previous results have shown clearly that only rats cured from carcinomatosis after treatment with apoptotic bodies/IL-2 developed antibodies against the cleaved form of BARD1 (p67; Ref. 13). This allows two nonexclusive explanations: anti-BARD1 antibodies contribute to the tumoral rejection; or their appearance is associated with treatment efficiency. Recent results from our laboratory indicate that the vaccination of rats with rat BARD1 F1 fragment (amino acids 460—611) leads to a 2-fold reduction of the tumor growth rate (data not shown). Although these results are preliminary, they illustrate the potential of BARD1 for the stimulation of an antitumoral immune response. Vaccination with p67 BARD1 cleavage product generated in vitro and fine mapping of sequences contributing to this antitumoral effect are the focus of ongoing research and should confirm our observations.

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