Autoubiquitination of BCA2 RING E3 Ligase Regulates Its Own Stability and Affects Cell Migration

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Abstract

Accumulating evidence suggests that ubiquitination plays a role in cancer by changing the function of key cellular proteins. Previously, we isolated *BCA2* gene from a library enriched for breast tumor mRNAs. The BCA2 protein is a RING-type E3 ubiquitin ligase and is overexpressed in human breast tumors. In order to deduce the biochemical and biological function of BCA2, we searched for BCA2-binding partners using human breast and fetal brain cDNA libraries and BacterioMatch two-hybrid system. We identified 62 interacting partners, the majority of which were found to encode ubiquitin precursor proteins including ubiquitin C and ubiquitin A-52. Using several deletion and point mutants, we found that the BCA2 zinc finger (BZF) domain at the NH$_2$ terminus specifically binds ubiquitin and ubiquitinated proteins. The autoubiquitination activity of BCA2, RING-H2 mutant, BZF mutant, and various lysine mutants of BCA2 were investigated. Our results indicate that the BCA2 protein is strongly ubiquitinated and no ubiquitination is detected with the BCA2 RING-H2 mutant, indicating that the RING domain is essential for autoubiquitination. Mutation of the K26 and K32 lysines in the BZF domain also abrogated autoubiquitination activity. Interestingly, mutation of the K232 and K260 lysines in and near the RING domain resulted in an increase in autoubiquitination activity. Additionally, in cellular migration assays, BCA2 mutants showed altered cell motility compared with wild-type BCA2. On the basis of these findings, we propose that BCA2 might be an important factor regulating breast cancer cell migration/metastasis. We put forward a novel model for BCA2 E3 ligase−mediated cell regulation. (Mol Cancer Res 2008;6(9):1385−96)

Introduction

The ubiquitin and ubiquitin-like pathways are integral to the normal function of eukaryotic cells (1-8). Protein turnover, trafficking, and the modulation of protein function have been ascribed to ubiquitination (9-11). Defects in ubiquitination programs have been described in the pathogenesis of several human diseases, including cancer.

Ubiquitination of target proteins proceeds in a stepwise format involving E1, E2, and E3 enzymes. In the first step, ubiquitin is activated in an ATP-dependent manner by the activating enzyme known as E1. In the second step, the activated ubiquitin is transferred to a conjugating enzyme denoted as E2. In the last step, the E2 enzyme interacts with a specific E3 ubiquitin-protein ligase resulting in the autoubiquitination of E3 or ubiquitination of target proteins on specific lysine residues (reviewed extensively in refs. 12-14). Target proteins can be ubiquitinated in a manner that alters their function, localization, or stability within the cell. Generally, the addition of one to four ubiquitin molecules to a target protein leads to a change in its localization and/or function. The addition of many ubiquitin moieties (polyubiquitination) leads to protein degradation by the 26S proteasome. Specificity in targeting proteins for ubiquitination lies mostly in the E3 enzyme. There are two major classes of E3 enzymes, the first being the HECT-type E3 ubiquitin ligases (15). These include Smurf1, Smurf2, AIP4, and Nedd4 among others. The second class of E3 enzymes is the RING finger domain proteins. For example, ring finger proteins Mdm2, Efp, and BRCA1/BARD have ubiquitin E3 ligase activity and have been shown to have a significant biological role in breast and other cancers (1, 13, 16, 17). A notable feature of RING E3 ubiquitin ligases is that the enzymatic activity of the E3 ligase can be monitored through autoubiquitination of the protein *in vitro*. autoubiquitination is the process by which the E3 enzymes catalyze the addition of polyubiquitin to themselves. This can result in the degradation or change of function of the E3 protein *in vivo*.

We identified BCA2, a RING finger protein that is overexpressed in >50% of invasive breast cancers compared with normal tissues and can be linked to breast cancer cell proliferation *in vitro* (16, 18). Overexpression of BCA2 increases the proliferation of NIH3T3 fibroblasts, whereas small interfering RNA inhibits the growth of BCA2-expressing breast cancer cells. BCA2 is expressed in the nucleus and cytoplasm of breast cancer cells, suggesting multiple functions (16). The BCA2 protein possesses autoubiquitination activity, which depends on its RING domain. Although it is well accepted that E3 enzymes could play an integral role in the oncogenic process (16, 19, 20), it is less well understood how deregulation of an E3 might occur.
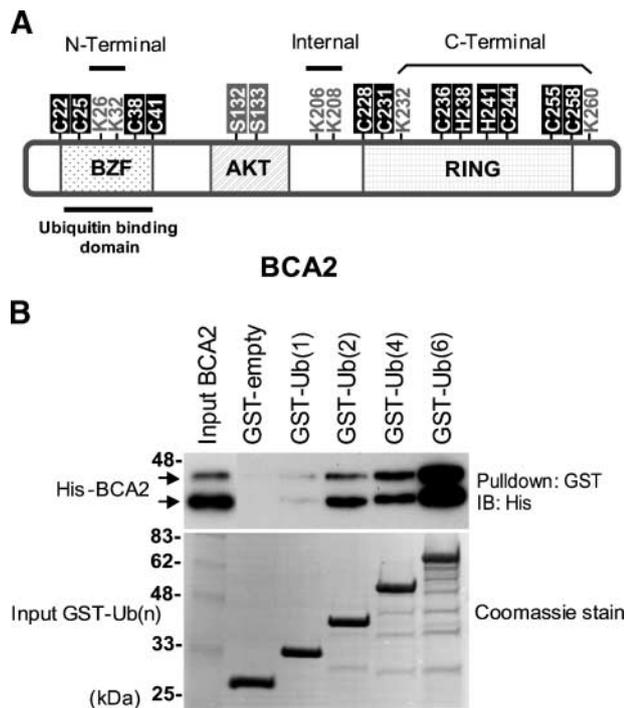
FIGURE 1. **A.** Schematic diagram of BCA2. Gray letters, positions of the six lysine residues: NH2-terminal lysines (*K26* and *K32*), internal lysines (*K206* and *K208*), and COOH-terminal lysines (*K232* and *K260*). Letters on black background, conserved cysteine (*C22, C25, C38,* and *C41*) residues in the BZF, which we show as the functional ubiquitin-binding domain, and cysteine and histidine (*C228, C231, C236, H238, H241, C244, C255,* and *C258*) residues in the RING domain. Letters on gray background, AKT phosphorylation sites (*S132* and *S133*). **B.** Recombinant BCA2 binds ubiquitin. GST-Ub(*n*) (1, 2, 4, or 6 ubiquitin tandem repeats) immobilized on glutathione sepharose beads were incubated with recombinant His-tagged BCA2. The pull-down assays were immunoblotted with anti-His antibody (*top*). The Coomassie brilliant blue R-250 staining of SDS-PAGE gel shows the amount of GST-Ub(*n*) fusion proteins used in each pull-down assay (*bottom*).

To gain a better understanding of how RING E3 enzymes mediate target identification and ubiquitination, we investigated the molecular mechanisms that regulate BCA2 E3 ligase activity. The BCA2 protein contains 304 amino acids and the protein sequence is conserved in various species (mouse, fruit fly, sea urchin, and fission yeast; ref. 16). Highly conserved regions include the zinc finger domain and the RING-H2 finger, suggesting important functions for these domains (Fig. 1A). Other regulatory motifs include a consensus AKT phosphorylation site and we have shown that BCA2 protein can be phosphorylated by AKT (21). We showed that BCA2 has E3 ligase activity by exhibiting its ability to undergo autoubiquiti-

nation *in vitro* using bacterially expressed recombinant protein. Like other E3 ubiquitin ligases, the ubiquitination activity of BCA2 is abolished by the mutation of two essential zinc-coordinating cysteine residues in the RING domain (16). A closely related protein, RNF126, has also been cloned and shares 46% identity with BCA2. We have shown that RNF126 also possesses autoubiquitination activity (22).

Our laboratory and others have shown that BCA2 binds to Rab7, a member of the Rab family of small G proteins that regulate intracellular vesicle traffic, including exocytosis and endocytosis (22, 23). Interestingly, there is some evidence that overexpression of BCA2 may down-regulate the epidermal growth factor receptor (23). We and others have found that Rab7 is not a substrate for BCA2 ligase activity (data not shown; ref. 24). Therefore, we sought BCA2-binding partners using the BacterioMatch system and human breast and fetal brain cDNA libraries. Interestingly, the majority of the positive clones were found to encode ubiquitin precursor genes including ubiquitin C and ubiquitin A-52, suggesting that BCA2 also binds noncovalently to ubiquitin and/or ubiquitin-modified proteins. Using various deletion and point mutations of BCA2, we identified a novel ubiquitin-interacting domain in the BCA2 protein and the major lysine residues required for the autoubiquitination activity. Furthermore, we show that alteration in the autoubiquitination activity of the BCA2 protein affects both the protein's steady state levels and BCA2-mediated cellular function. On the basis of our findings, we propose a novel mechanism for BCA2 E3 ubiquitin ligase activity and its role in cell regulation.

Results

Characterization of BCA2 Partner Proteins

To isolate BCA2-binding partners, we used the Bacterio-Match II system and the human breast and fetal brain cDNA libraries. Three million clones were screened and 62 were found to be strongly positive after tertiary screening. Interestingly, all of the 61 genes encode proteins that function within the ubiquitin-proteasome pathway. Fifty of the positive clones encoded ubiquitin C, 10 of them the ubiquitin A-52 ribosomal fusion protein (Uba52), and 1 clone corresponded to Ubc9 (Table 1). To confirm the interaction between ubiquitin and BCA2, we used various lengths of concatamerized ubiquitin moieties (Ub_1 to Ub_6) fused to recombinant glutathione-*S*-transferase (GST) in a pull-down assay with recombinant BCA2 protein. We show that the BCA2 protein specifically binds to all GST-tagged ubiquitin fusion proteins, but not to GST alone (Fig. 1B). Notably, the strongest binding was observed with the Ub_6 ubiquitin, which contains six tandem repeats of the ubiquitin protein.

Table 1. Characteristics of the Genes Which Tested Positive Following Bacterial Two-Hybrid Tertiary Screening of a Human Fetal Brain Library Using BCA2 Wild-Type Protein as Bait

Accession No.	Insert (kb)	Clone/ORF (Amino Acids)	Gene Name
NP_066289	0.5-1.8	85-480/685	Polyubiquitin C
NM_003333	2.8	128/128	Ubiquitin-52 amino acid fusion protein (UBA52)
NM_003345	1.2	158/158	Ubiquitin-conjugating enzyme E2 (Ubc9)
NM_000980	0.6	176/176	60S ribosomal protein L18A

Table 2. Sequence Comparison of the BZF Domain and Various Zinc Finger Type Ubiquitin Binding Domains

Protein	Domain	Amino Acid Sequence
HDAC6	PAZ	VTQP C GD C GTIQENWV - - - - C LS C YQVY C GRYING H
Np14	NZF	AMWA C QH C TFMNQPGTGH - - C EM C SLPRT
Polη	UBZ	DQVP C EK C GSLVPVWDMPE H MDY H FALELQKS
Rabex5	A20	SELL C KKG C YYGNPAWQGF C SK C WREEYHKA
BCA2	BZF	H R FF C H F C KGE V SP K LPE Y I C PR C ESGFIEE V
RNF126	BZF	G R YF C HC C S VE I VP R LPD Y I C PR C ESGFIEE L

NOTE: Amino acid sequence were taken from PAZ (25), NZF (26), UBZ (27), and A20 (28). Letters on black background, conserved cysteine and histidine residues for zinc finger domains; open boxes, conserved amino acids within BZF domains of BCA2 and RNF126; dashes, gaps inserted to maximize conserved amino acid alignment.

Identification of a Novel Ubiquitin-Interacting Domain in BCA2

Towards the NH₂-terminal region of the protein (residues 22-41), BCA2 contains a single zinc finger (CxxC-CxxC). We have termed the BCA2 zinc finger domain as BZF. At least four other zinc finger domains (PAZ, NZF, UBZ, and A20) have been shown to interact with ubiquitin (Table 2; refs. 25-30). We hypothesized that the BCA2 zinc finger may also interact with ubiquitin. To test this hypothesis, we tested several BCA2 deletion variants in a directed bacterial two-hybrid assay (Fig. 2A). Figure 2B shows that only the wild-type BCA2 and the NH₂-terminal fragment mutant, which retains the complete BZF domain, resulted in colony growth but not with an internal or COOH terminal fragment mutant indicating that the interaction of BCA2 with ubiquitin C or Uba52 requires the BZF domain.

To further confirm that the BCA2 BZF domain is indeed a functional ubiquitin-binding motif, we employed a modified pull-down approach in which we used wild-type or site-directed BZF domain mutants of BCA2 in which cysteine residues of the BZF zinc finger were substituted with alanines (Fig. 2C). RING domain and AKT site point mutants were used as controls (Fig. 2C). Using ubiquitin-conjugated beads, we showed that wild-type BCA2 and only the mutants that retained the wild-type BZF domain interact with ubiquitin (Fig. 2D).

Quantification of BZF Binding Affinity for Ubiquitin

To better understand the binding affinity of the BZF domain for ubiquitin, we conducted a quantitative association study to determine the K_d for this interaction. Surface plasmon resonance (SPR) biosensors analyze macromolecular interactions in
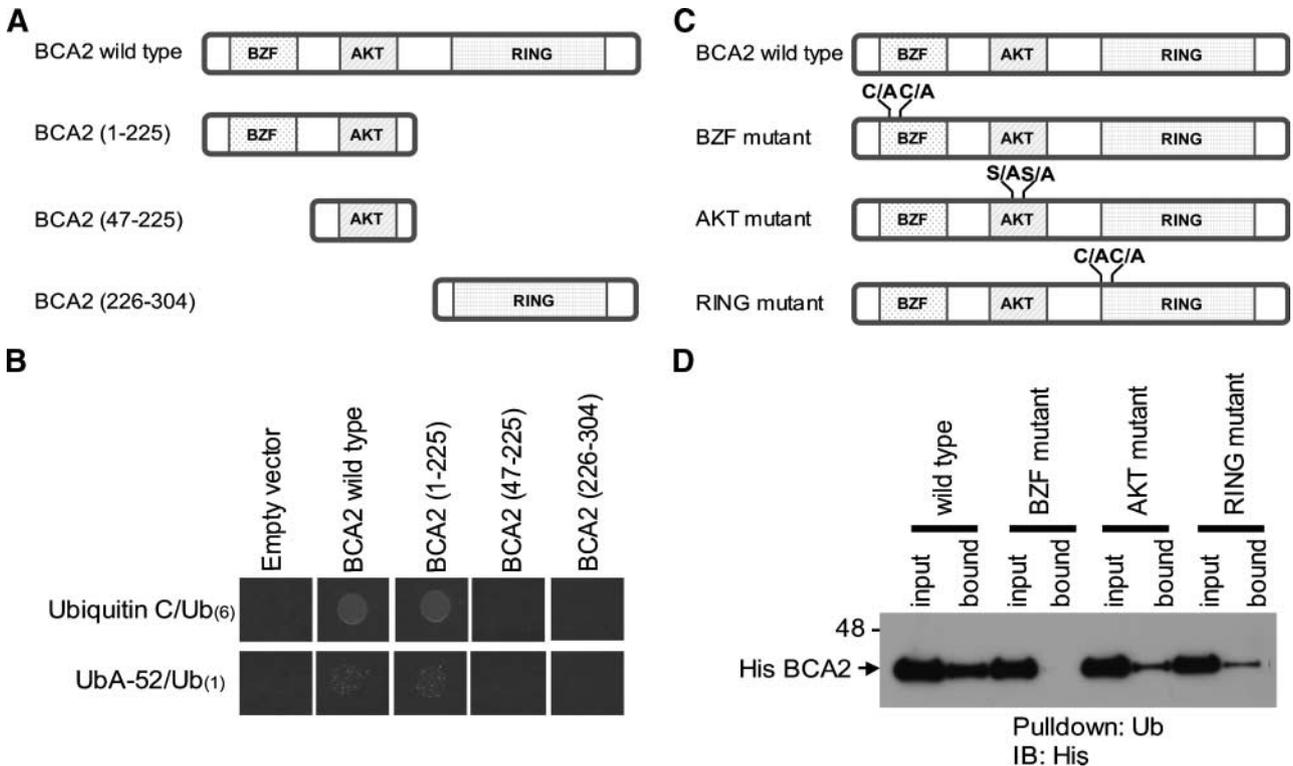

FIGURE 2. BCA2 interacts with ubiquitin via the BZF domain. **A.** Schematic diagram of BCA2 and its deletion mutants used for directed bacterial two-hybrid assay. **B.** Bacterial cells expressing the BCA2 wild-type or NH₂-terminal fragment (1-225) of BCA2 interact with both six tandem ubiquitin and UbA-52 ribosomal fusion protein grown in a selective plate containing 3-AT and streptomycin, whereas pBT empty vector, BCA2 internal fragment (47-225), and COOH-terminal fragment (226-304) failed to interact with ubiquitin. **C.** Schematic diagram of BCA2 and its point mutants used for pull-down assay in **D**. **D.** Recombinant his-tagged BCA2 and its BZF, AKT, and RING mutants were subjected to pull-down assays with GST-Ub(4) immobilized on glutathione sepharose beads. GST-Ub(4) bound to BCA2 wild-type, AKT, and RING mutants but not to the BCA2 BZF mutant.

real-time without the need to label the substrates. To quantify the BCA2-ubiquitin interactions by SPR, a layer of BCA2 was adsorbed onto the surface of the sensor chip and a solution of ubiquitin was subsequently flowed over the surface-bound BCA2 for binding. As a control, we used the zinc-ejecting compound, disulfiram, which we have shown to disrupt the BZF domain (16). When the BZF domain is disrupted, either through zinc ejection by disulfiram or by specific mutation of cysteine to alanine in the BCA2 zinc finger (BZF mutant), we observed a loss of ubiquitin-binding to BCA2. We found that the BZF domain binds ubiquitin with a K_d of 29.6 ($\pm$3.2) μmol/L (Fig. 3A) and shows a much higher affinity when compared with other known ubiquitin-binding domains (Fig. 3B).

Autoubiquitination Activity of BCA2 Is Dependent on Its RING Domain

Autoubiquitination is a characteristic of RING E3 ligases (31, 32), and in the absence of a specific target, autoubiquitination can be used to monitor E3 enzymatic activity. Furthermore, autoubiquitination may represent a significant

FIGURE 3. Quantification of BZF domain affinity for ubiquitin. **A.** Determination of the dissociation constant by measuring the amount of ubiquitin bound to BZF domain of BCA2 using SPR. Ubiquitin protein was injected in triplicate at concentrations of 0 to 1,000 mmol/L over His-tagged BCA2 immobilized on CM5 sensor chip (■, K_d = 29.6 $\pm$ 3.2 mmol/L). A much weaker binding (K_d) response was obtained when the same concentrations of ubiquitin were injected over BCA2 treated with the zinc-ejecting compound, disulfiram (▲). Fitted curves are shown (*bars,* SD based on three independent experiments). **B.** Comparison of binding affinity of BCA2-BZF domain and various distinct ubiquitin-binding domains, UEV (34), UBA (35), UIM (36), NZF (26), and A20 (28). All binding affinities were measured by SPR.

means by which E3 ligases autoregulate their own stability within the cell. It is therefore important to understand the mechanisms by which autoubiquitination occurs. E3 autoubiquitination can be evaluated in an *in vitro* autoubiquitination assay, in which the E3 is mixed with its cognate E2 and the other factors required for the ubiquitination reaction.

The actual E2 enzymes that BCA2 coordinates with for autoubiquitination are UbcH5s. Using either UbcH5a, UbcH5b, or UbcH5c, we evaluated BCA2 autoubiquitination activity through an *in vitro* ubiquitination assay. We showed that wild-type BCA2 is autoubiquitinated in the presence of UbcH5b; however, in the absence of a cognate E2, BCA2 lacks autoubiquitination activity (Fig. 4). Like other RING E3 ligases, the enzymatic activity responsible for BCA2 autoubiquitination requires an intact RING finger domain. Because BCA2 loses its autoubiquitination activity when the cysteines C228 and C231 within the RING finger are mutated, indicating that the first two cysteine residues in the RING finger domain are necessary for E3 ligase activity. However, the AKT phosphorylation site mutant displayed modest autoubiquitination activity.

Kinetics of BCA2 Autoubiquitination

The effects of various BCA2 mutants on autoubiquitination kinetics were examined in time course autoubiquitination assays. Aliquots of *in vitro* reactions after 15, 30, 60, and 90 minutes were immunoblotted to reveal ubiquitinated BCA2 protein (Fig. 5). Densitometry values normalized to background were plotted and graphed for each BCA2 variant tested (Fig. 6). Wild-type BCA2 produced detectable ubiquitination after 60 minutes, increasing after 90 minutes (Figs. 5A and 6, *orange square*). No effect was observed when we used the AKT site mutant of BCA2 (Figs. 5B and 6, *purple triangle*). In contrast, mutation of essential cysteines in the BZF domain increased the amount of ubiquitinated BCA2 protein after 60 and 90 minutes (Figs. 5C and 6, *dark blue square*). As we have shown above and in ref. (16), the ubiquitination activity is completely abolished by mutation of the RING domain (Figs. 5D and 6, *green triangle*).

Mutation of Lysine and Cysteine Residues Affects Autoubiquitination of BCA2

Ubiquitination occurs on lysine residues. BCA2 possesses six lysine residues that are located almost in pairs at the NH2-terminal, middle, and COOH-terminal regions of the protein. To elucidate which lysine residues are responsible for autoubiquitination, we created arginine mutants for all six BCA2 lysines. The two NH2-terminal lysines are located in the BZF domain and we denote these two residues as the NH2-terminal lysines (K26 and K32). The next two lysines are located between the AKT phosphorylation site and the RING finger domain and we denote these two residues as the internal lysines (K206 and K208). The last two lysines are located within and just next to the RING finger domain. We denote these last two residues as the COOH-terminal lysines (K232 and K260; Fig. 1A).

In an autoubiquitination time course assay, mutation of the NH2-terminal lysines (K26R and K32R) located within the BZF region resulted in a complete lack of ubiquitinated BCA2, indicating that these are the most likely sites of BCA2 autoubiquitination (Figs. 5E and 6, *orange square*). However,
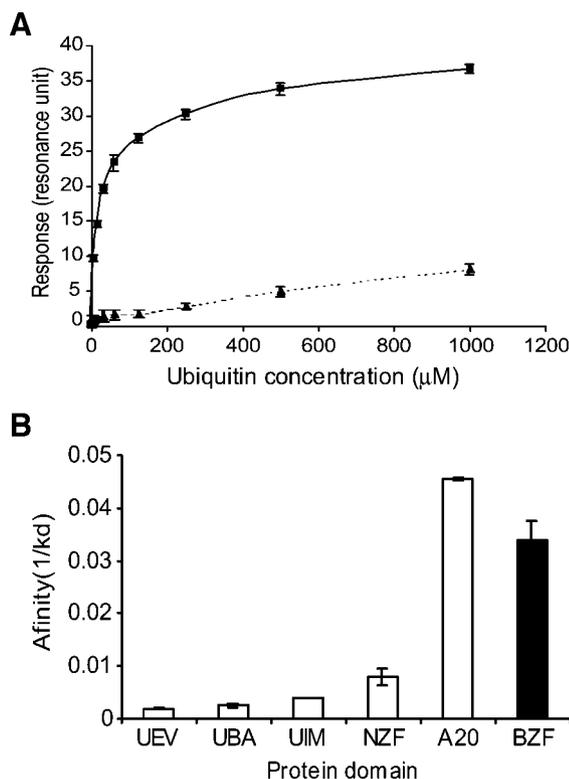
FIGURE 4. Autoubiquitination of the BCA2 proteins. BCA2 and its mutants were expressed using the pCMV-tag2B-Flag vector or pEBG-GST vector in HEK293T cells. Flag-tagged proteins were immunoprecipitated (*IP*) with monoclonal anti-Flag antibodies and incubated with protein A sepharose beads. GST-tagged proteins were pulled-down with glutathione sepharose 4B. Washed beads were incubated with ubiquitin, ATP, mouse E1, and with (+) or without (−) the bacterial E2 UbcH5b. Anti-Flag immunoblot of autoubiquitination assay for Flag-tagged BCA2 (*left*). Anti-GST immunoblot of autoubiquitination assay for GST-tagged BCA2 (*right*).
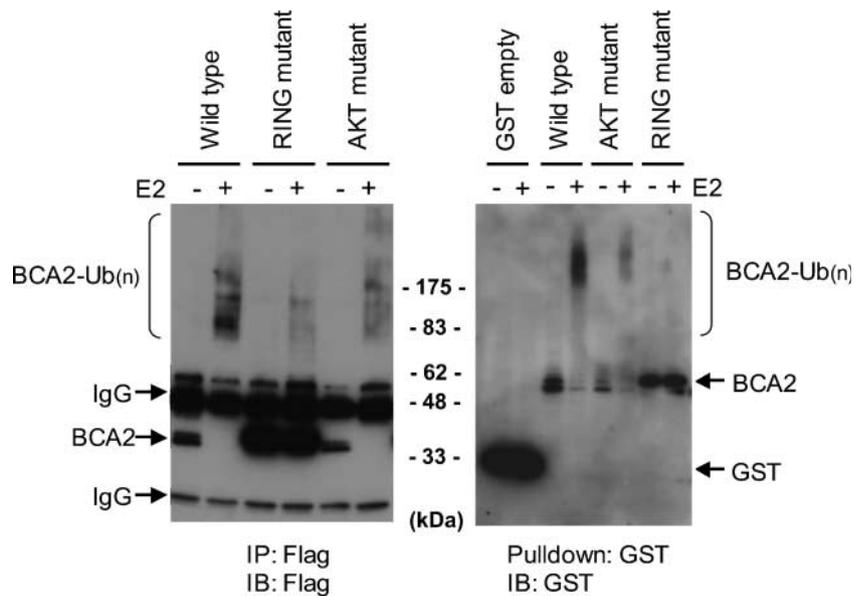
individual mutations of the NH_2-terminal lysines produced ubiquitinated BCA2, indicating that either of these lysines (K26R and K32R) can be used for autoubiquitination (Figs. 5F and 6, *red triangle* and Figs. 5G and 6, *green triangle*). Mutation of the internal lysines (K206R/K208R) produced autoubiquitinated BCA2 protein after 60 minutes (Figs. 5H and 6, *blue triangle*). Interestingly, mutation of the two COOH-terminal lysines (K232R/K260R) located within and nearby the RING domain increased both the amount and the kinetics of BCA2 ubiquitination reaction (Figs. 5I and 6, *purple square*). Further refinement of this observation was done using single COOH-terminal lysine mutants of BCA2. Mutation of COOH-terminal lysine K232 showed a slight decrease in autoubiquitination compared with wild-type BCA2 (Figs. 5J and 6, *dark blue triangle*). BCA2 autoubiquitination was greatly enhanced by mutation of the COOH-terminal lysine K260R (Figs. 5K and 6, *blue square*). Lastly, we combined mutations that resulted in an increase in BCA2 autoubiquitination, i.e., the BZF and COOH-terminal lysine mutants. Here, we observed further increases in BCA2 autoubiquitination (Figs. 5L and 6, *red square*). As a result, it seems that disruption of the BCA2 BZF domain or COOH-terminal K260 lysine increases both the amount and the rate of BCA2 autoubiquitination. From the kinetic analyses, we were able to subdivide different BCA2 variants into four groups: ligase dead, ligase normal, ligase high, and ligase very high (Fig. 6).

BCA2 Autoubiquitination Activity Correlates with Its Own Stability

Recently, we had shown that BCA2 steady state protein levels could be stabilized by the addition of proteasome inhibitor MG-132 (16). This work also showed that mutation of the RING domain prevented proteasomal degradation of the BCA2 protein, indicating a possible autoubiquitination-mediated autoregulation mechanism for the BCA2 protein. In this study, we compared the steady state levels of wild-type, lysine mutants, BZF domain mutants, and RING mutants of BCA2 in

HEK293T cells (Fig. 7). Mutation of the BZF domain cysteines and mutation of the COOH-terminal lysines (K232R/K260R) located within and nearby the RING domain resulted in decreased amounts of BCA2 protein relative to wild-type protein (Fig. 7, *lanes 7* and *11* versus *lane 1*). Because mutation of lysines within and near the RING domain reduces BCA2 stability, this indicates that these mutations do not affect the function of the RING domain but do increase the rate of BCA2 protein degradation. This occurred in both the absence and in the presence of the proteasome inhibitor MG-132 (Fig. 7, *lanes 7* and *8*), suggesting that autoubiquitination and degradation of BCA2 is markedly enhanced by mutations of the BZF domain and the COOH-terminal lysines. As expected, autoubiquitination-deficient NH_2-terminal lysine mutants and RING finger mutant did not show an increase in BCA2 degradation, which is in agreement with our ubiquitination studies. Our observations indicate that the BCA2 RING is required for BCA2 autoubiquitination and that the two NH_2-terminal lysines are the most likely sites for polyubiquitination of the BCA2 protein.

Enhanced BCA2 E3 ligase activity is associated with an increase in cellular motility in MCF7 breast cancer cells. To better understand the role of BCA2 in cell migration, we generated various BCA2 and its variants expressing MCF-7 cell lines. The expression of exogenous BCA2 protein in these cell lines was examined by *in situ* immunofluorescence (Fig. 8) and Western blotting assays (Fig. 9A). Immunofluorescence was carried out as described in Materials and Methods. The wild-type BCA2 protein showed diffused expression throughout the cell with small areas of intense staining (Fig. 8A), the RING mutant showed similar patterns as wild-type BCA2, however, it was expressed at much higher levels (Fig. 8D). The NH_2-terminal lysine mutant showed punctate staining in the cytoplasm and near the cell membrane (Fig. 8B). A similar speckled expression pattern was observed for the COOH-terminal lysine mutant and the BZF mutant (Fig. 8C and E). We also determined the expression levels by Western blotting and found that the

proteins of the autoubiquitination-dead versions of BCA2 (RING and NH_2-terminal lysine mutants) were stabilized and expressed at a higher level than the wild-type BCA2 protein. On the other hand, the BZF and COOH-terminal lysine mutants, which have very high autoubiquitination activities, appeared at lower levels than the wild-type BCA2 protein (Fig. 9A).

The scratch wound assay was used to assess the effects of BCA2 and its mutants on the cellular migration associated with wound closing. MCF-7 cells stably expressing different BCA2 variants were plated to confluence in a six-well plate. A single scratch wound was created in each well using the pipette tip, and cells were treated with hydroxyurea to block cellular proliferation. The wound area within each well was imaged at fixed time intervals (Fig. 9B). We quantified the net migration of BCA2 expressing MCF7 cells with three independent points in each experiment using imaging software (see Materials and Methods) and the data is plotted as bar graphs (Fig. 9C). We discovered that the MCF-7 cells expressing the wild-type BCA2 protein were significantly more motile than control cells. The BZF domain mutant showed the most migration of all the MCF-7 BCA2-expressing stable lines tested, the wound was
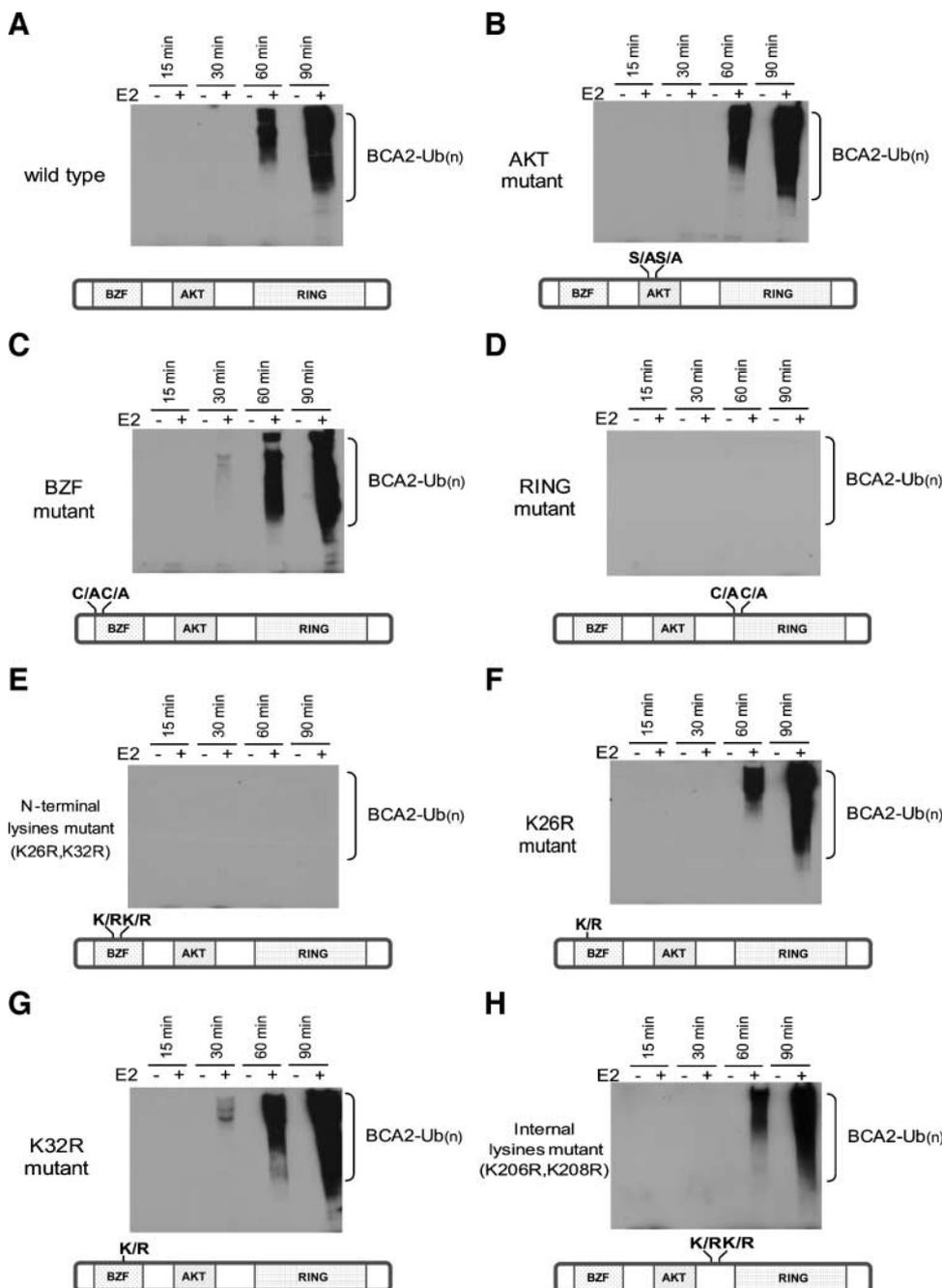

FIGURE 5. Time course for the detection of BCA2 and its mutant autoubiquitination. Time course of *in vitro* ubiquitination reactions (15, 30, 60, and 90 min) conducted in the presence (+) or absence (−) of bacterial extract containing the E2-conjugating enzyme UbcH5b and purified ubiquitin with E1 protein, using bacterially expressed His-BCA2 (**A**), His-BCA2 AKT mutant (**B**), His-BCA2 BZF mutant (**C**), His-BCA2 RING mutant (**D**), His-BCA2 NH_2-terminal lysine (K26R, K32R) mutant (**E**), His-BCA2 K26R mutant (**F**), His-BCA2 K32R (**G**), His-BCA2 internal lysine (K206R, K208R) mutant (**H**), His-BCA2 COOH-terminal (K232R, K260R) mutant (**I**), His-BCA2 K232R mutant (**J**), His-BCA2 K260R mutant (**K**), and His-BCA2 BZF + COOH-terminal (K232R, K260R) mutant (**L**). Autoubiquitination assay conditions are described in Materials and Methods.
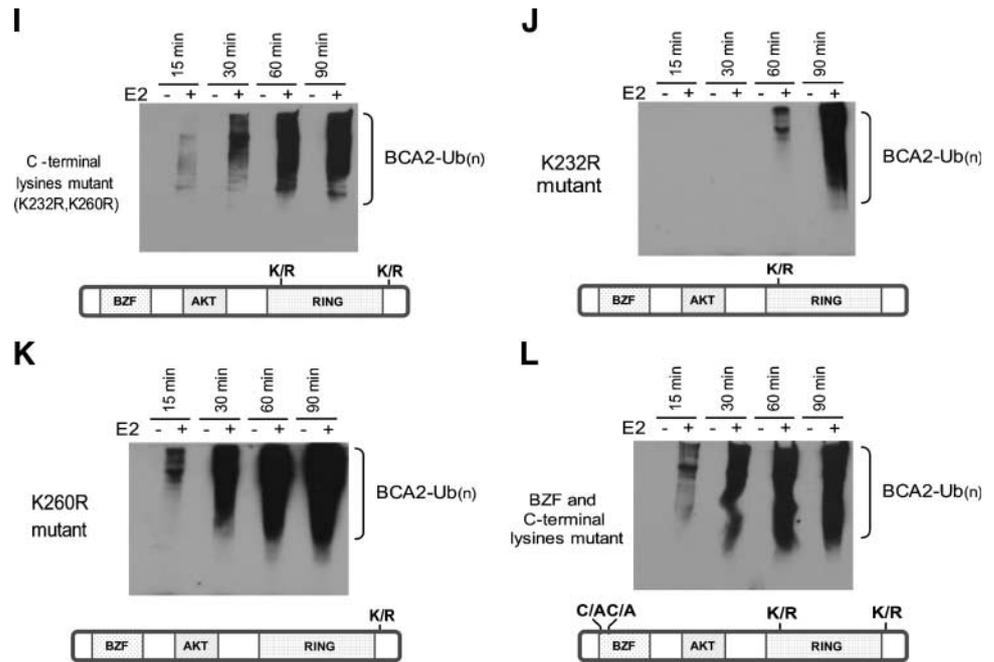
FIGURE 5 Continued.

75% closed at the 24-hour time point and was completely closed after 48 hours. In contrast, mutants that abolished autoubiquitination (RING mutation or NH_2-terminal lysine mutations) showed decreased mobility and <20% wound closure was seen after 24 hours.

Discussion

RING E3 ligases play pivotal roles in protein degradation and receptor-mediated endocytosis. BCA2 is a RING finger protein possessing ubiquitination ligase activity (16). Originally isolated by us through subtractive hybridization cloning from breast cancer cells, we later showed that BCA2 is overexpressed in invasive breast cancers and is a potential target for therapeutic interventions (16, 18, 33). BCA2 E3 ligase is coregulated with estrogen receptor and plays a role in the regulation of epidermal growth factor receptor trafficking. The biological significance of BCA2 overexpression also lies in the area of cellular transformation, as ectopic BCA2 increased breast cancer cell line proliferation and small interfering RNA inhibited the growth of cultured cells (16). The mechanism for these effects likely lies in its intrinsic ubiquitination activity (16). This was further illustrated when we examined stably expressing MCF-7 cell lines of BCA2 and various BCA2 mutants. We showed that overexpression of BCA2 or BCA2 mutants that have an increase in autoubiquitination activity also show an increase in cell migration when tested in scratch assays (Fig. 9).

The autoubiquitination activity requires the presence of an E2 as we showed with UbcH5s (Fig. 4). We showed that not all UBCs coordinate with BCA2, as autoubiquitination is not detectable when UBC3 is used in the assays (data not shown). The autoubiquitination activity of BCA2 also depends on the RING domain, as BCA2 with a mutated RING domain lacking key cysteines is not ubiquitinated *in vitro* (Fig. 5) and only shows extremely low levels of ubiquitination *in vivo* (Fig. 4). We believe that the very low levels of BCA2 RING mutant autoubiquitination detected *in vivo* could be due to the presence of endogenous factors that might cooperate with BCA2 autoubiquitination activity. It is also possible that the ring mutant has residual autoubiquitination activity that is detectable only when the BCA2 is expressed in a mammalian system. In this study, we have also identified a novel zinc finger domain in BCA2, which is responsible for binding ubiquitin noncovalently. We termed this new domain as BZF and provided evidence for the function of the BZF domain in the regulation of BCA2 autoubiquitination activity (Fig. 5).

In order to identify targets of ubiquitination by BCA2, we identified BCA2 partner proteins using the BacterioMatch two-hybrid assay system (Table 1). The specific targets included ubiquitin, ubiquitinated proteins, and Ubc9. The interaction between these proteins and BCA2 was confirmed in a directed two-hybrid assay and Western blotting of immunocomplexes. Using mutants of BCA2, we confirmed that ubiquitin bound BCA2 in the BZF domain. The noncovalent interaction between ubiquitin and BCA2 suggests that the BCA2 ubiquitin ligase activity might be directed to other proteins in addition to itself. Owing to its activity as an E3 ubiquitin ligase and our understanding of the essential role of the ubiquitin proteasome system in protein homeostasis, BCA2 might influence tumorigenesis through the regulation of cancer-related proteins. BCA2 autoubiquitination seems to be a tightly controlled process because we found that overexpression of hyperactive autoubiquitination mutants (K232R/K260R) in HEK293T cells resulted in an almost complete absence of the BCA2 protein (Fig. 7). Point mutations that negatively affect BCA2 autoubiquitination
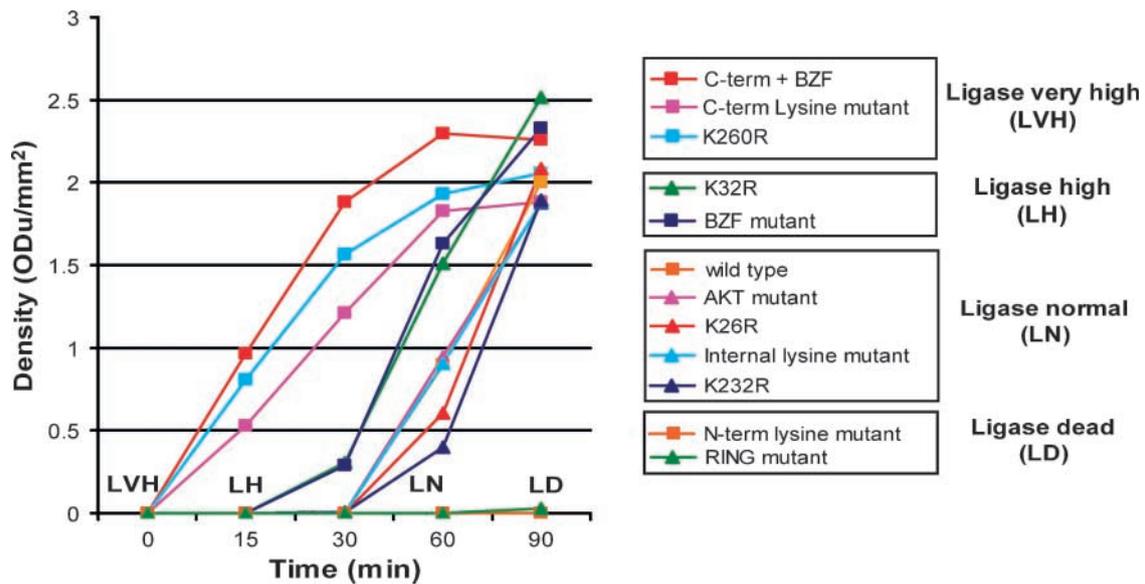
FIGURE 6. Comparison of E3 ligase activity of BCA2 and its mutants. The absorbance of individual bands of each Western blot (**A-L**) was quantitated using Quantity One software (ver. 4.3.1, Bio-Rad Laboratories) and normalized by subtracting the absorbance of an area of identical size from the blank lane of each gel.

activity such as the RING domain mutant (which is catalytically dead) or the NH_2-terminal lysine mutant (which cannot receive polyubiquitination) result in the accumulation of the BCA2 protein (Fig. 7). This observation indicates that BCA2 undergoes a self-regulated life span within the cell. The analysis of lysine mutants in BCA2 allowed us to find the sites of autoubiquitination, and to better understand novel mechanisms by which BCA2 may regulate target specificity. We found that the lysines contained within the BZF domain were required for BCA2 autoubiquitination. Furthermore, cysteine to alanine mutation of the BZF domain, which disrupts the zinc finger, led to an increase in autoubiquitination. This observation may indicate a mechanism whereby the BCA2 protein binds monoubiquitinated substrates via the

BZF domain for further ubiquitination or for intracellular trafficking/targeting. However, when substrate levels drop or when BCA2 levels are relatively high, BCA2 will autoubiquitinate, thereby regulating its levels according to substrate availability (Fig. 10). Indeed, we observed strong differences in subcellular localization between the different mutants of BCA2, most notably the NH_2-terminal lysine mutant and BZF mutant seem to have strong punctate patterns of expression compared with the wild-type BCA2 protein (Fig. 8). These results suggest that ubiquitination of BCA2 and the ubiquitin-binding activity are important determinants for BCA2 stability and localization within the cell. This may represent a general mode of autonomous self-regulation for some of the E3 ligases.
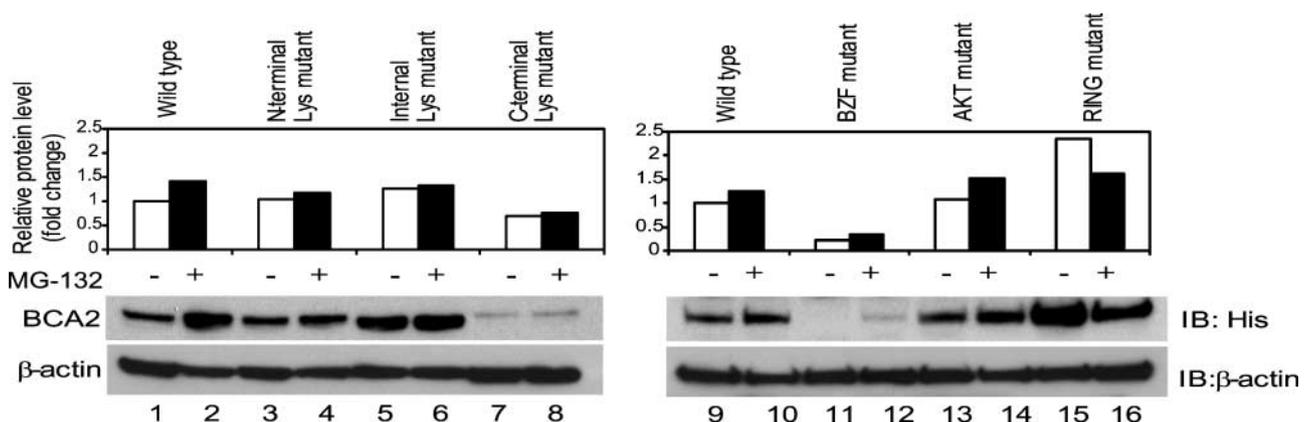

FIGURE 7. Stability of transiently expressed BCA2 wild-type and its mutants. Equal amounts of His-tagged BCA2 wild-type and its mutant vector DNA were transfected into HEK293T cells. The total transfection period was 48 h; 10 mmol/L of MG-132 (+) or DMSO (−) were added 40 h after transfection, resulting in drug exposure of 8 h. Whole cell lysates were immunoblotted with anti-His (*top*) or β-actin was used as a loading control (*bottom*). Bar graph, normalized fold protein level of BCA2 and its mutants relative to BCA2 wild-type MG-132 (−) using β-actin as internal control.

Materials and Methods

Antibodies

The mouse monoclonal anti-FLAG (M2), anti–GST-2, and anti–β-actin (AC-15) antibodies were purchased from Sigma. The monoclonal antipolyhistidine (AD1.1.10) antibody was from R&D Systems. The monoclonal antiubiquitin (P4D1) antibody was from Cell Signaling Technologies. Horseradish peroxidase–conjugated antimouse IgG secondary antibody was from Promega.

Bacterial Two-Hybrid Screening

A screen for BCA2-interacting proteins was done using the BacterioMatch II two-hybrid system (Stratagene). Full-length human BCA2 cDNA was cloned as a *Not*I-*Sal*I fragment from FLAG-tagged pCMV-BCA2 (16) into the *Not*I-*Xho*I digested bait vector, pBT. The BacterioMatch human breast and fetal brain cDNA libraries constructed by pTRG vector were purchased from Stratagene. Cotransformants were screened by using medium lacking histidine and containing 5 mmol/L 3-amino-1,2,4-triazole, as recommended by the manufacturer. The plates were incubated at 37°C for 24 h and then at room temperature for 24 h to detect weak interactors. Positive

interactions were further confirmed by activation of the streptomycin resistance gene on selective plates. For negative controls, empty pBT and pTRG plasmids were cotransformed with pTRG library and pBT-BCA2, respectively. A positive control included the cotransformation of pBT-LGF2 and pTRG-Gal11, provided by Stratagene. DNA sequencing reactions of the respective inserts in pTRG vector were done at the Sunnybrook Research Institute Genomic Core Facility. A BLAST search at the National Center for Biotechnology Information web site was done to identify proteins encoded by the respective cDNAs.

Expression Vector Constructs and Recombinant Proteins

Histidine-tagged bacterial recombinant BCA2 and its mutant proteins were expressed in *Escherichia coli* strain BL21 star (DE3) using pET100/D-TOPO expression vectors and purified on nickel-charged agarose beads as described (16). Point mutations were generated in BCA2 coding region using the QuickChange site-directed mutagenesis kit from Stratagene. Ubiquitin (one, two, four, or six ubiquitin tandem repeats) cDNA were amplified from positive clones isolated from bacterial two-hybrid screening by PCR and inserted into the
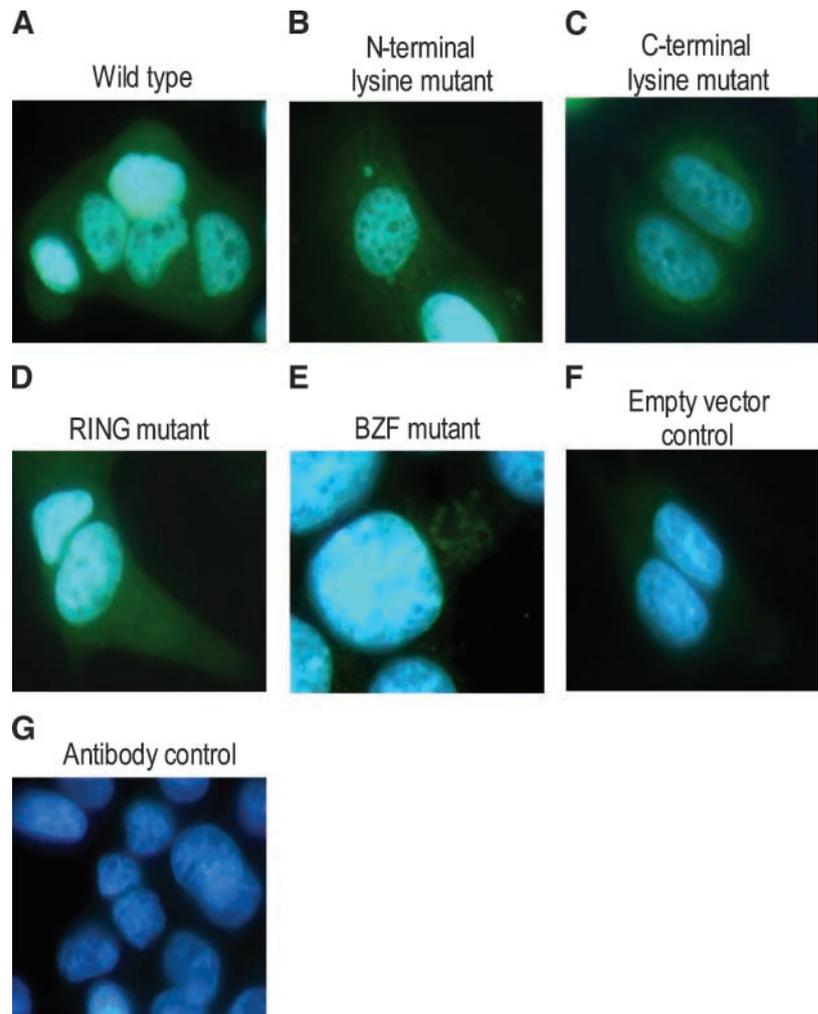

FIGURE 8. BCA2 and its variants expression in MCF7 cells. MCF7 cell lines that stably express BCA2 or selected mutant variants were examined by immunofluorescence as described in Materials and Methods. Fixed cells were probed with the monoclonal antipolyhistidine (AD1.1.10) antibody for 8 h followed by antimouse FITC-secondary fluorescent antibody. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole. *In situ* immunofluorescence reveals different expression patterns for the BCA2 protein variants. **A.** MCF7 cells expressing BCA2 wild-type protein. **B.** MCF7 cells expressing BCA2 NH₂-terminal lysine mutant protein. **C.** MCF7 cells expressing BCA2 COOH-terminal lysine mutant protein. **D.** MCF7 cells expressing BCA2 RING mutant protein. **E.** MCF7 cells expressing BCA2 BZF mutant protein. **F.** MCF7 cells expressing His tag alone (empty vector). **G.** Negative control in which only secondary FITC antibody was incubated with MCF7 cells expressing the His tag from the empty vector.
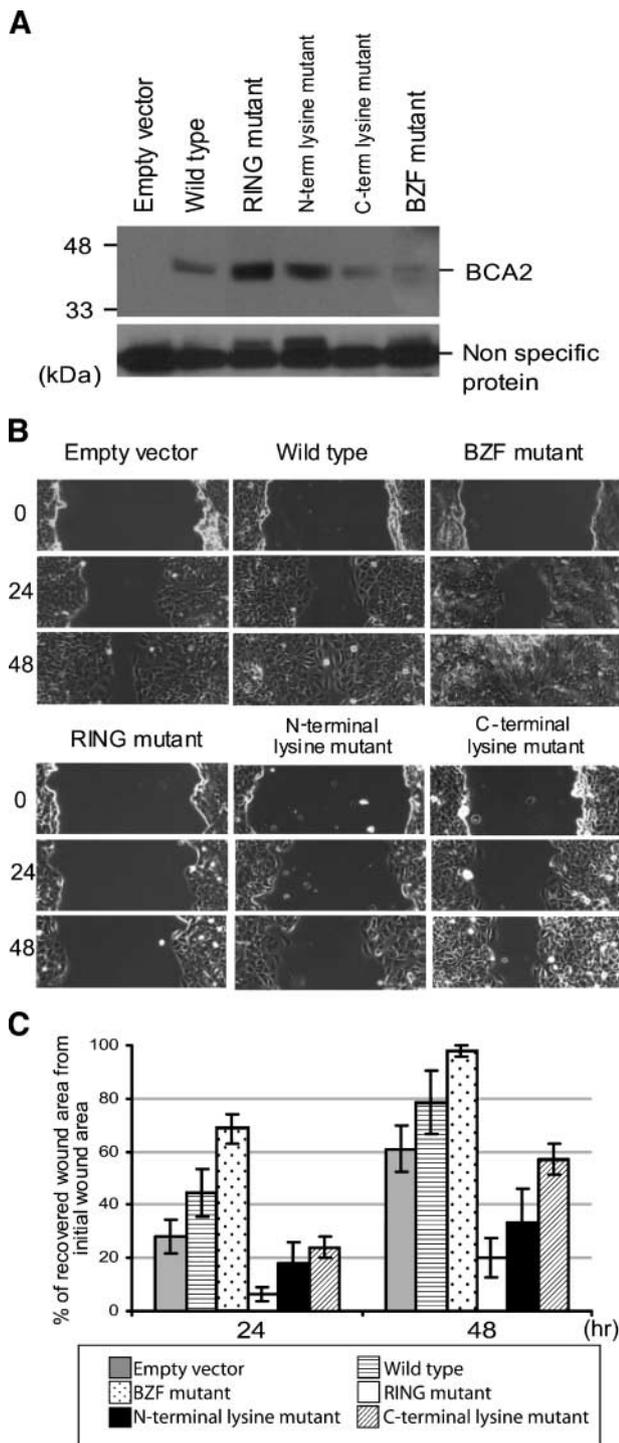
FIGURE 9. Effects of BCA2 and mutants on MCF7 cell migration. **A.** Anti-His immunoblot of MCF7 cells stably transfected with pEF1-His-BCA2 wild-type and its mutants (*top*). Nonspecific band obtained by anti-His was used as a loading control (*bottom*). **B.** MCF7 cells stably expressing BCA2 or its mutants were serum-starved for 18 h, scratched with a sterile pipette tip, and treated with DMEM containing 10% fetal bovine serum and 10 mmol/L of hydroxyurea. Cells were allowed to migrate into the denuded area, and phase micrographs were taken at 0, 24, and 48 h. **C.** Columns, percentage of recovered wound area from the initial wound area after 24 and 48 h of migration; bars, SD based on three independent experiments.

pGEX 4T3 expression vector (GE Healthcare). Expressed GST-tagged ubiquitins were isolated from crude bacterial extracts using glutathione sepharose 4B beads (GE Healthcare).

BCA2 Constructs for Expression in Mammalian Cells

A vector expressing GST-tagged BCA2 was created by inserting the *Bam*HI fragment of BCA2 cDNA from FLAG-tagged pCMV-BCA2 into the pEBG-GST vector. Plasmids expressing His-BCA2 and mutants were constructed in the pEF1 vector from pET100-BCA2 and its mutants.

Cell Culture and Transfection

HEK293T cells were maintained in high glucose DMEM (Life Technologies) containing 10% fetal bovine serum without antibiotics at $37°C/5\% CO_2$. Cells were plated in six-well plates the day before transfection was done. Typically, 4 μg of vector DNA is transfected into cells by using LipofectAMINE 2000 (Invitrogen) and proteins were extracted 48 h later, as described (16).

Ubiquitination Assays

Purified bacterially expressed His-BCA2 (100 ng), or Flag-BCA2 immunoprecipitated with anti-FLAG monoclonal antibody or GST-BCA2 pulled-down with glutathione sepharose 4B (GE Healthcare) from 1 mg of whole cell lysate of transfected HEK293T cells were analyzed for autoubiquitination activity. BCA2 or its mutants were mixed with 3 μL of 10× reaction buffer [500 mmol/L Tris-HCl (pH 8.0), 20 mmol/L DTT, and 50 mmol/L MgCl₂], 1 μL rabbit E1 (50 ng; Calbiochem), 1 μL ubiquitin (1 μg; Sigma), 1 μL E2 (50 ng, UbcH5b bacterial product), 3 μL of 20 mmol/L ATP and water, to obtain a final reaction volume of 30 μL. Each reaction mixture was incubated at 30°C for 90 min, and proteins were visualized by Western blotting. Films were scanned on a Universal hood (SN75S) densitometer and signals quantitated using the Quantity One software (ver.4.3.1; Bio-Rad Laboratories).

Analysis of Protein Stability

Four micrograms of wild-type or mutant His-BCA2 vector DNA was transfected into HEK293T cells. After 40 h, the proteasome inhibitor MG-132 (10 μmol/l) was added for a total of 8 h. Control cells were treated with vehicle (DMSO) for 8 h. Whole cell lysates were analyzed for protein expression levels by Western blotting.

Western Blotting

Samples were mixed with 3× SDS-loading buffer containing 10% β-mercaptoethanol, boiled for 10 min and loaded onto either 2% SDS-polyacrylamide gel or 4% to 20% Tris-HEPES SDS-polyacrylamide gel (Pierce) for electrophoretic separation. The proteins were transferred onto a Hybond-P polyvinylidene difluoride membrane (GE Healthcare) at 15 V overnight. All procedures after blotting were done at room temperature. The membrane was blocked for nonspecific binding with 5% milk powder in TBST (TBS with 0.2% Tween 20; pH 7.4) for 1 h. Primary antibodies were diluted in blocking buffer and incubated for 2 h on a shaker. Membranes

Proposed Model for BCA2 Regulation

FIGURE 10.   Proposed model for BCA2 regulation.
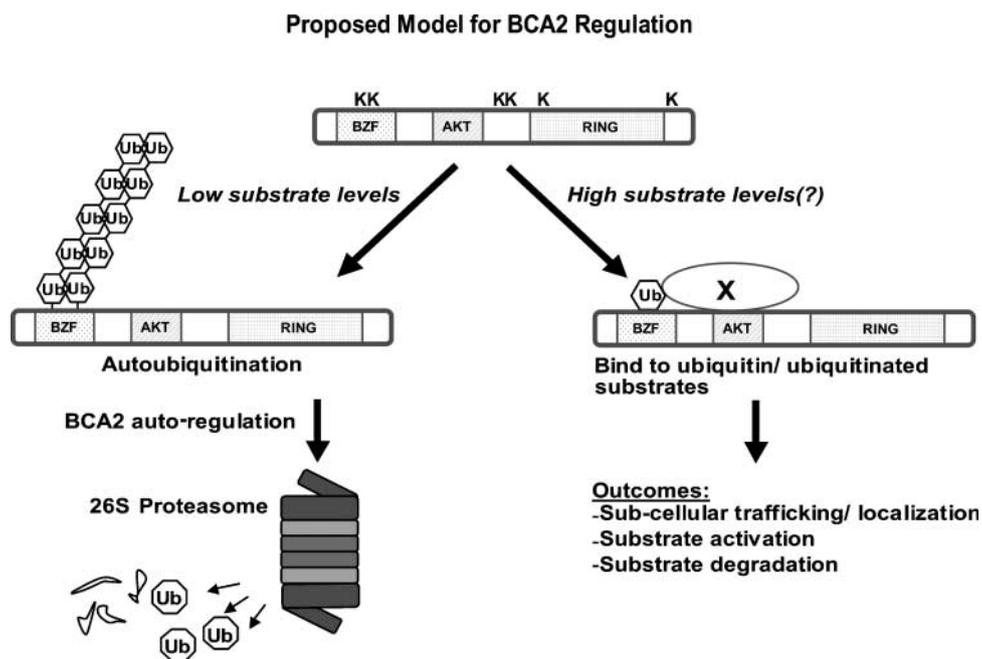
were then washed twice with TBST. Afterwards, each membrane was incubated with species-specific secondary antibody conjugated to horseradish peroxidase enzyme for 1 h, washed thrice with TBST, and the signals were developed by the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce) and by exposure to X-ray film (Kodak).

SPR Binding Assay

SPR measurements were done on a BIACORE 3000 equipped with a research-grade CM5 sensor chip at 25°C with a flow rate of 50 μL/min. Bacterially expressed and purified His-tagged BCA2 or zinc finger mutant (negative control) were immobilized at final densities of 0.8 to 1.0 kRU using traditional amine-coupling chemistry. Ubiquitin in running buffer (HBS-P) was injected in triplicate over the His-tagged BCA2 and zinc finger mutant surfaces at concentrations of 0, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, and 1,000 μmol/L (50 s at 50 μL/min). Between subsequent injections of ubiquitin, surfaces were regenerated with an injection of HBS-P supplemented with 5 mmol/L of DTT and 30 μmol/L of $ZnCl_2$ for 6 min at 50 μL/min. Binding responses were recorded and globally fit to simple 1:1 binding models. The structure of the BZF domain was disrupted with an injection of 10 μmol/L disulfiram, the potent zinc-ejecting compound, for 10 min (16).

Immunofluorescence

MCF7 clones stably expressing wild-type or mutant BCA2 or empty vector were grown on glass coverslips for 24 h. Cells were fixed with 4% paraformaldehyde and blocked with 10% goat serum overnight at 4°C. Fixed and blocked cells were then probed with the monoclonal antipolyhistidine (AD1.1.10) antibody (R&D Systems) for 8 h at 4°C. Washes were done with PBS and repeated thrice. Antimouse FITC-secondary

fluorescent antibody was obtained from Sigma and used according to the manufacturer's specifications. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole. Final washes were done with PBS and repeated five times. Coverslips were mounted on slides and observed in a Zeiss Axiovert 200M system.

Wound Healing Assay

Individual MCF7 clones expressing wild-type or mutant BCA2 were seeded in six-well culture plates and grown to confluence forming a monolayer covering the surface of the entire well. After cells were serum-starved in serum-free DMEM for 18 h, the wound was created in the center of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. Debris was removed by PBS wash, and the cells received fresh DMEM with 10% fetal bovine serum and 10 mmol/L of hydroxyurea. Four individual optical images of the wound edges were taken by the Zeiss Axiovert 200M microscope at 24 and 48 h. The ability of the cells to migrate into the wound area was quantified by measuring the number of pixels in each wound closure area using Adobe Photoshop. Data are expressed as the percentage of the wound closure from the initial wound area.

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Autoubiquitination of BCA2 RING E3 Ligase Regulates Its Own Stability and Affects Cell Migration

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