BRCA1 Affects Lipid Synthesis through Its Interaction with Acetyl-CoA Carboxylase*

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Germ line alterations in BRCA1 (breast cancer susceptibility gene 1) are associated with an increased susceptibility to breast and ovarian cancer (1). Germ line mutations of BRCA1 are found in about 50% of patients with inherited breast cancer and up to 90% of families with breast and ovarian cancer susceptibility (1, 2). Frequent loss of the wild-type allele in tumors of BRCA1 mutation carriers suggests that BRCA1 acts as a tumor suppressor gene. However, although BRCA1 has been implicated in a number of cellular processes, such as transcription, DNA repair, and ubiquitination, the molecular mechanisms responsible for tumorigenesis are not yet fully understood. We have recently demonstrated that BRCA1 interacts in vivo with acetyl coenzyme A carboxylase α (ACCA) through its tandem of BRCA1 C terminals (BRCT) domains. To understand the biological function of the BRCA1-ACCA complex, we sought to determine whether BRCA1 is a regulator of lipogenesis through its interaction with ACCA. We showed here that RNA inhibition-mediated down-regulation of BRCA1 expression induced a marked increase in the fatty acid synthesis. We then delineated the biochemical characteristics of the complex and found that BRCA1 interacts solely with the phosphorylated and inactive form of ACCA (P-ACCA). Finally, we demonstrated that BRCA1 affects lipid synthesis by preventing P-ACCA dephosphorylation. These results suggest that BRCA1 affects lipogenesis through binding to P-ACCA, providing a new mechanism by which BRCA1 may exert a tumor suppressor function.

BRCA1 (breast cancer susceptibility gene 1) was the first susceptibility gene linked to breast and ovarian cancer (1). Germ line mutations of BRCA1 are found in about 50% of patients with inherited breast cancer and up to 90% of families with breast and ovarian cancer susceptibility (1, 2). Frequent loss of the wild-type allele in tumors of BRCA1 mutation carriers suggests that BRCA1 acts as a tumor suppressor gene. However, although BRCA1 has been implicated in a number of cellular processes including the DNA damage repair (3), the biological role of BRCA1 remains unclear.

The BRCA1 gene encodes an 1863-amino acid protein with a tandem of two BRCA1 C terminus (BRCT)5 domains at its C-terminal region (4–6). The majority of disease-associated BRCA1 mutations result in a truncated product with loss of one or two BRCT domains (2). BRCT domains function as protein-protein interaction modules (7). Recently, it has been shown that a subset of tandem of BRCT domains, including those of BRCA1, function as phosphopeptide-binding modules (8–10). We previously identified acetyl-CoA carboxylase α (ACCA) as a novel partner of BRCA1 (11). Notably, the binding of ACCA is mediated by the tandem of BRCT domains of BRCA1, and it is abolished by tumor-associated mutations that affect these domains. Thus, the in vivo interaction of ACCA and BRCA1 is likely to be important for BRCA1-mediated tumor suppression. ACCA is the rate-limiting enzyme for long chain fatty acid synthesis that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA (12). Malonyl-CoA is then used for long chain fatty acid synthesis by fatty acid synthase (FAS). A number of critical ACCA phosphorylation sites have been identified (13). More specifically, phosphorylation on Ser79 by the AMP-activated protein kinase (AMPK) is responsible for the inactivation of ACCA (14).

To explore new functions of BRCA1 that may contribute to its tumor suppressor activity, we investigated functional features of the BRCA1-ACCA complex (11). We first examined the biological properties of BRCA1 with respect to those of ACCA and found that BRCA1 silencing increases cellular lipid synthesis. We then explored the biochemical characteristics of the complex BRCA1-ACCA: BRCA1 interacts solely with the phosphorylated and inactive form of ACCA (P-ACCA), and the formation of the BRCA1-P-ACCA complex interferes with ACCA activity by preventing P-ACCA dephosphorylation. Therefore, we identified a new function of BRCA1 that is to modulate lipid synthesis through its phospho-dependent binding to ACCA.

EXPERIMENTAL PROCEDURES

Chemicals—The primary antibodies used were as follows: monoclonal antibody to human BRCA1 (OP92) (Oncogene Research Products), monoclonal antibody to actin (ICN Biochemicals), polyclonal antibody to ACCA (ACCAL3) as previously described (11), and polyclonal antibody to P-ACCA (P-ACCA (S79)) (Upstate Biotechnology). The specificity of the P-ACCA(S79) antibody was tested by pretreatment of the antibody with the phosphopeptide immunogen and by pretreating the blot with phosphatase (Upstate Biotechnology). Secondary antibodies were peroxidase-conjugated anti-mouse or anti-rabbit

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5 The abbreviations used are: BRCT, BRCA1 C terminus; ACCA, acetyl coenzyme A carboxylase α; FAS, fatty acid synthase; AMPK, AMP-activated protein kinase; SCD-1, stearoyl-CoA desaturase-1; SREBP-1, sterol regulatory element-binding protein-1; LXR, liver X receptor; ACAR, S-aminooimidazole-4-carboxamide riboside; AIPase, k protein phosphatase; RT, reverse transcription; GST, glutathione S-transferases; siRNA, small interfering RNA; RNAi, RNA inhibition; P-ACCA, phosphorylated and inactive form of ACCA.
immunoglobulins (Amersham Biosciences). 5-Aminomidazole-4-carboxamide riboside (AICAR) was purchased from Toronto Research Chemicals. A protein phosphatase (PPase) was purchased from Biolabs.

Expression Vectors—The pCDNA3β plasmid expressing human BRCA1 protein was previously described (15). The ACCA sequence corresponding to N-terminal (4019 bp) fragment (ACCA-N) was PCR-amplified from human cDNA library, using respectively the primers Nte-F 5′-GGTCAAGGATATGAACTGATCATC-3′ and Nte-R 5′-AAGGGGCGCTGTCACTGAGGAAATAG-3′. The cDNA fragment was subcloned into the XhoI/NotI sites of pCDNA3.1/Myc-His (Invitrogen) and sequenced. The ACCA-N/ST9A mutant was generated by site-directed mutagenesis (QuickChange XL site-directed mutagenesis kit; Stratagene) with the following primers: 5′-CATAGGTCAAGCATGCGTTAGCCATCT TAG-3′ and 5′-CTAGGTGCAAGGACCATGCTGAGG3′- and sequenced.

Cell Culture and Plasmid Transfections—MCF7 human breast cancer cells were maintained in Dulbecco’s modified Eagle’s medium containing 1 g/liter of glucose supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml of penicillin. When indicated, 0.5 mM of AICAR was added to cell medium. Bosc human embryonic kidney cells were maintained in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter of glucose supplemented as above. MCF10A normal mammary epithelial cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented as described (16).

For transfection, MCF7 cells were plated at 4 × 10⁵ cells/plate in a 6-well flask 24 h before transfection. The cells were transfected with 2 μg of plasmid and 10 μl of ExGen 500 (Euromedex) following the supplier procedure. 48 h after transfection, the cells were processed for immunoblotting or metabolic labeling. Bosc cells were transfected with 4 μg of plasmid and 20 μl of ExGen 500 (Euromedex) following the supplier procedure. 48 h after transfection, the cells were lysed. The lysates were pooled and then divided in the desired number of samples that were further processed for immunoprecipitation.

Immunoprecipitation and Immunoblotting—The cells were lysed in lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40, 10% glycerol) supplemented with protease inhibitors (Complete EDTA free; Roche Applied Science) and phosphatase inhibitors (1 mM glycerophosphate, 50 mM sodium fluoride, and 1 mM sodium orthovanadate). Immunoprecipitation and immunoblotting were performed as described (11). Where indicated, the intensity of the bands corresponding to the immunoreactive proteins were quantified using Quantity One software (Bio-Rad).

GST Pull-down Assay—The cells were lysed in lysis buffer supplemented with protease and phosphatase inhibitors (as above). Where indicated as “No stress,” the cells were lysed in culture dish and scraped afterward. Where indicated as “AICAR,” the cells were treated for 1 h with 0.5 mM of AICAR followed by lysis in culture dish and scraping. Otherwise, the cells were treated with trypsin before lysis. GST pull-down assay was performed as described (11). The presence of GST fusion proteins was examined by Coomassie Blue staining. Where indicated, the protein complexes were treated with 40 units of APase for 1 h at 30°C, before analysis by Western blotting. Alternatively, GST pull-down was performed on cell lysates previously treated with 2000 units of APase.

RNAi—MCF7 cells were plated at 5 × 10⁶ cells/plate in a 6-well flask 24 h before transfection with small interfering RNA (siRNA). The siRNA duplexes (Proligo, France) were provided as purified and annealed duplexes. The sequences were as follows: Si-BRCA1 5′-AAAGGACUGUCUCCAAAAGUU-3′ and Si-control 5′-AACACGAGUGACAGUAUUU-3′. The DNA sequences were subjected to a BLAST analysis. The cells were transfected with 0.125 nmol of siRNA and 3 μl of Lipofectamine 2000 (Invitrogen) using the protocol of the supplier. The cells were processed for RT-PCR, immunoblotting, or metabolic labeling 72 h after transfection.

Quantitative Reverse Transcription PCR—Total RNA was isolated using the RNeasy Mini kit (Qiagen). After extraction, the integrity and quantity of RNA were examined by gel electrophoresis. Total RNA was quantified by densitometry on a 1.2% agarose gel with known amount of standard RNA (Roche Applied Science).

The RT-PCR assay was performed by coamplification of 0.3 μg of total RNA and of a known amount of competitor RNA. Primers used were designed to amplify the cDNA fragment from exons 6 to 8 of the BRCA1 gene (C3F, 5′-TGCTGTTTTCAGCTTGAACCAAG-3′, and C3R, 5′-CGTCTTTTGAAGTTGGATACGGC-3′) (17). The reactions were performed using the One Step RT-PCR kit (Qiagen). The procedure used was as described by the supplier. The aliquots were analyzed on a 2% agarose gel containing 0.1 μg/ml ethidium bromide, and the intensity of the bands corresponding to the wild-type BRCA1 PCR products and to the competitor PCR products were quantified using Quantity One software (Bio-Rad). A PCR using the same primers was realized on total RNA to confirm the absence of DNA in the RNA samples (17).

Real Time RT-PCR—Total RNA was prepared as above. The real time RT-PCR was performed mainly as described (18). First strand cDNAs were first synthesized from 1 μg of total RNA in the presence of 100 units of Superscript II (Invitrogen) using both random hexamers and oligo(dT) primers (Promega). Real time PCR was performed in a final volume of 20 μl containing 5 μl of a 60-fold dilution of the RT reaction medium, 15 μl of reaction buffer from the fastStart DNA Master SYBR Green kit (Roche Applied Science), and 10.5 pmol of the specific forward and reverse primers (Eurobio, France). The list of the primers and real time PCR conditions for each mRNA assay are available upon request. For quantification, a standard curve was systematically generated with six different amounts (150–30,000 molecules/tube) of purified target cDNA cloned in the pGEM plasmid (Promega). Each assay was performed in duplicate, and validation of the real time PCR runs was assessed by evaluation of the melting temperature of the products and by the slope and error obtained with the standard curve. The analyses were performed using Light-Cycler software (Roche Applied Science). The results are presented as relative levels after normalization by hypoxanthine phosphoribosyltransferase-1 mRNA abundance.

Metabolic Labeling—2-14C-Labeled acetate (53 mCi/mmol; 2 μCi/dish) (Amersham Biosciences) was added to the culture medium. After 4 h of incubation at 37°C, the cells were washed with phosphate-buffered saline and harvested, and the lipids were extracted according to Folch’s technique (19). Radioactivity was measured by scintillation counting. The values were normalized for sample protein contents. Acetate incorporation into specific lipids was analyzed after separation of lipids by TLC. Therefore, lipid extracts and appropriate lipid standards were spotted on silica gel (60 Å; Whatman). For separation of lipids, the plates were developed in hexane-diethyl ether-acetic acid (70/30/1, v/v/v). The lipid samples and standards were visualized by coloration in staining solution (0.12 m NaCl, 20% methanol, 300 mg/liter Coomassie Blue). The gel lipid fractions were resuspended into 50% methanol solution, and radioactivity was measured by scintillation counting. The values were normalized for sample protein contents.

E. Lefai, personal communication.
FIGURE 1. BRCA1 silencing up-regulates fatty acid synthesis in MCF7 cells. MCF7 cells were transfected with control siRNA (Si-control) or siRNA directed against BRCA1 (Si-BRCA1). 72 h after transfection, the cells were treated as follows. A, total RNAs were purified. A quantitative RT-PCR analysis was performed from 0.3 μg of total RNA mixed with various amount of competitor molecules. Lane 1, 5 × 10^6 molecules; lane 2, 1 × 10^6 molecules; lane 3, 5 × 10^5 molecules; lane 4, 1 × 10^5 molecules. For each panel, 5 μl of RT-PCR (total volume, 50 μl) were analyzed on a 2% agarose gel containing ethidium bromide (upper panel). The intensity of the bands corresponding to PCR products were quantified using Quantity One software (Bio-Rad) to determine the quantity of BRCA1 mRNA molecules in each samples. The values represent the means ± S.E. of experiments performed in triplicate. *, p < 0.05, compared with Si-control (lower panel).

B, BRCA1 protein was detected by immunoblotting analysis using OP92 antibody. ACCA protein immunoblotting using ACCAL3 antibody was used as an internal control (upper panel). The intensity of bands corresponding to BRCA1 was quantified, and the values are presented on the diagram. The values represent the means ± S.E. of experiments performed in triplicate. *, p < 0.05, compared with Si-control (lower panel).

C, cells were treated with 2-14C-labeled acetate for 4 h, and radioactivity was measured from lipid extracts. The values were normalized for sample protein contents. The values represent the means ± S.E. of experiments performed in quadruplicate. ***, p < 0.001 compared with Si-control. D, cells were treated with 2-14C-labeled acetate for 4 h, and the different lipid species were separated by TLC; 14C incorporation was measured by
Statistical Analysis—Results are expressed as the means ± S.E. Statistical analysis was performed with a two-tailed paired Student’s t test. For the analysis of lipid synthesis, the statistical significance of the results was determined using the nonparametric one-tailed Mann-Whitney test. The threshold for significance was set at \( p < 0.05 \).

RESULTS

**BRCA1 Affects Fatty Acid Synthesis**—To investigate the role of BRCA1 in modulating fatty acid synthesis, we used a siRNA approach on MCF7 cells. Inhibition of endogenous BRCA1 expression after transfection with the BRCA1-specific siRNA oligonucleotide (Si-BRCA1) was analyzed by a quantitative RT-PCR assay using a chimeric BRCA1 RNA as a competitor (17) and by Western blotting. RNAi reduced the expression of BRCA1 mRNA by 73% and BRCA1 protein by 65% compared with MCF7 cells transfected with the control siRNA (Si-control), with no effect on the amount of ACCA protein (Fig. 1, A and B).

To assess potential effects of RNAi-mediated BRCA1 silencing on lipid synthesis, MCF7 cells transfected with Si-BRCA1 or Si-control siRNAs were exposed to 2-14C-labeled acetate. Incorporation of 2-14C-labeled acetate into cellular lipids of MCF7 cells transfected with Si-BRCA1 was significantly increased to 150 ± 3% (mean ± S.E.; \( n = 4 \); \( p < 0.001 \)) of the level in the cells transfected with control siRNA (Fig. 1C).

To investigate the impact of RNAi-mediated BRCA1 silencing on the different lipid species, the lipid extracts were analyzed by TLC. The majority of 2-14C-labeled acetate in cells was incorporated into phospholipids, as previously described (20, 21). BRCA1 down-regulation caused 2-14C-labeled acetate incorporation into phospholipids to significantly increase by \( \sim 1.7 \)-fold (\( p = 0.025, z = 1.96 \)) (Fig. 1D). Free fatty acid synthesis was also largely increased with a clear significance (2.79 ± 0.48-fold, \( p = 0.023, z = 1.99 \)), and triglyceride synthesis was increased by 1.59 ± 0.35 with a near limit significance (\( p = 0.075, z = 1.44 \)). In contrast, no significant increase was found regarding the slight differ-

scintillation counting. The values represent the means ± S.E. of experiments performed in triplicate. *\( p < 0.05 \) compared with Si-control (Mann-Whitney test). E, measurement of SREBP-1a, SREBP-1c, LXRα, LXRβ, ACCA, FAS, and SCD-1 expression in MCF7 cells. The histograms represent mRNA levels determined using real time RT-PCR and are expressed as relative levels (hypoxanthine phosphoribosyltransferase-1 mRNA as a reference). The values represent the means ± S.E. of experiments performed in triplicate. LXRα, LXRβ, LXRδ; SREBP-1c, SREBP-1c, SREBP-1c, SREBP-1c, SREBP-1c, SREBP-1c; values were multiplied by 1000 for homogeneous representation. The threshold for significance was set at \( p < 0.05 \). No significant differences were found between Si-control (open columns) and Si-BRCA1 (filled columns) transfected MCF7 cells. F, analysis of expression of ACCA and FAS proteins in MCF7 cells. BRCA1 was detected by immunoblotting using OP92 monoclonal antibody; ACCA and FAS were analyzed by immunoblotting using polyclonal antibodies from the same cell lysates. Actin was used as an internal control. WB, Western blot.
ence of cholesterol synthesis (1.3-fold, \( p = 0.25, z = 0.65 \)). It is noteworthy that this slight difference of cholesterol synthesis fits in with recent studies reporting that ACCA RNAi slightly reduced the synthesis of cholesterol, whereas FAS RNAi did not (22). Taken together, our findings concerning different lipid species are in agreement with the above increase into total cellular lipids (Fig. 1C). Furthermore, the fact that BRCA1 knockdown increased lipogenesis through fatty acid synthesis independently of cholesterol synthesis strongly sustains the notion that BRCA1 acts through modulation of lipogenesis.

To verify that the increase in lipogenesis was not due to transcriptional up-regulation of lipid synthesis enzymes, we examined gene expression of proteins involved in lipogenesis. We first assessed expression of SREBP-1 transcription factor, a master regulator of lipogenesis (23). Measurement of SREBP-1a and SREBP-1c transcripts using real time RT-PCR showed a small difference in cells transfected with Si-BRCA1. Because this slight decrease was not significant \( (p = 0.11 \) and \( p = 0.19, \) respectively) and was not consistent with the increase of lipogenesis obtained in Si-BRCA1-transfected cells, it likely does not account for increase in lipogenesis (Fig. 1F). We then analyzed the expression of key lipogenic enzymes ACCA and FAS, both highly regulated by SREBP-1, and the downstream enzyme SCD-1, also SREBP-1-regulated. As expected, the amounts of ACCA, FAS, and SCD-1 mRNAs were not significantly modified by BRCA1 silencing \( (ACCA: \ p = 0.48, FAS: \ p = 0.08; SCD-1: \ p = 0.94) \) (Fig. 1E). Finally, because SREBP-1, FAS and SCD-1 are also regulated by LXR receptors, we analyzed expression of LXRe and LXR\( \beta \) (24). As anticipated, no significant difference was found regarding amounts of LXRe and LXR\( \beta \) (Fig. 1E).

We also examined the expression of ACCA and FAS proteins. Western blot analysis using anti-ACCA and anti-FAS antibodies showed that the expression of both proteins was not affected by BRCA1 silencing \( (Fig. 1F) \). Collectively these data show that the increase in lipogenesis was independent of transcriptional regulation.

To further ascertain the role of BRCA1 in lipogenesis, MCF7 cells were transfected with expression plasmid encoding BRCA1 and were exposed to 2-\(^{14}\)C-labeled acetate. In these cells, expression of BRCA1 protein was significantly increased to 250 \( \pm \) 13% \( (mean \ \pm \ S.E.; \ n = 3; \ p < 0.001) \) without affecting the amount of ACCA protein \( (Fig. 2, A \ and \ B) \). In these cells that overexpressed BRCA1, incorporation of 2-\(^{14}\)C-labeled acetate into cellular lipids was significantly decreased to 51 \( \pm \) 2% \( (mean \ \pm \ S.E.; \ n = 5; \ p < 0.001) \) of the level in control cells \( (Fig. 2C) \). Therefore, overexpression of BRCA1 efficiently repressed lipogenesis, to the same extent as BRCA1 silencing increased it, thus confirming the role of BRCA1 in lipogenesis.

We also analyzed the impact of RNAi-mediated gene silencing of BRCA1 on untransformed breast epithelial cells MCF10A. Similar to MCF7 cells, siRNA targeting of BRCA1 decreased the expression of BRCA1 mRNA and BRCA1 protein without affecting the expression of ACCA \( (Fig. 3, A \ and \ B) \); incorporation of 2-\(^{14}\)C-labeled acetate into cellular lipids was significantly increased in comparison with the control cells \( (Fig. 3C) \). Altogether, these results show that down-regulation of BRCA1 induces an increase in cellular lipid synthesis independently of a transcriptional regulation and imply that endogenous BRCA1 negatively influences fatty acid synthesis.
BRCA1 Associates with Phosphorylated ACCA—Our previous observations that BRCA1 interacts with ACCA (11) prompted us to further investigate how BRCA1 affects fatty acid synthesis through this interaction. It has been shown that phosphorylation and dephosphorylation of ACCA cause its inactivation and activation, respectively, and serve as the short term regulation mechanism of the enzyme. More specifically, inactivation of ACCA is driven by AMPK-mediated phosphorylation on Ser79 (14). Therefore, to explain how BRCA1 negatively modulates fatty acid synthesis, we tested whether BRCA1 associates with phosphorylated and inactive form of ACCA, namely P-ACCA. For this purpose, we used the P-ACCA(S79) antibody purchased from Upstate Biotechnology.

To first ascertain the specificity of this antibody toward Ser79, we assayed it in Western blotting and immunoprecipitation assays. Western blotting experiments performed on Bosc cell lysates treated with APase that release phosphate groups from serine, threonine, and tyrosine residues in proteins showed that this antibody did not recognize the dephosphorylated form of ACCA, unlike ACCAL3 antibody (Fig. 4A, left panel). In a more specific approach, Western blotting was performed on Bosc cells transfected with expression plasmid encoding the N-terminal moiety of ACCA (ACCA-N) or its form specifically mutated on Ser79 to Ala79 (ACCA-N-S79A). As expected, P-ACCA(S79) antibody did not detect the mutated N-terminal moiety of ACCA (ACCA-N-S79A), whereas it detected the wild-type ACCA-N. As control, ACCAL3 antibody detected both ACCA-N and ACCA-N-S79A forms (Fig. 4A, left panel). For immunoprecipitation experiments, the specificity of the antibody was checked using Bosc cells transfected as above. P-ACCA(S79) antibody immunoprecipitated only the nonmutated ACCA-N form, whereas ACCAL3 immunoprecipitated both forms (Fig. 4A, right panel). Therefore, the specificity of the P-ACCA(S79) antibody for phosphorylated form of ACCA on Ser79 was clearly demonstrated to support its use in the following experiments.

To test whether BRCA1 associates with P-ACCA, two approaches were carried out. First, GST pull-down assays using a GST-BRCT fusion protein were performed on MCF7 cells. Western blotting analysis using the P-ACCA(S79) antibody clearly showed that the BRCA1 BRCT domains bound endogenous P-ACCA (Fig. 4B). Second, to determine whether BRCA1 interacts with P-ACCA in vivo, lysates prepared from Bosc cells transfected with expression plasmid encoding full-length BRCA1 were immunoprecipitated with P-ACCA(S79) antibody and analyzed by immunoblotting. As shown in Fig. 4C, faint signals were obtained with the controls (a nonimmune serum and an assay performed in the absence of antibody) (lanes 2 and 3), in contrast to the marked signal obtained with P-ACCA(S79) antibody (lane 4). We ensured that the quantity of IgG used in each immunoprecipitation reaction was similar by performing Western blotting using secondary anti-rabbit IgG antibody (lanes 3 and 4). Therefore, BRCA1 associated with endogenous P-ACCA in mammalian cells. In the reciprocal immunoprecipitation we could not detect P-ACCA in association with BRCA1, this was possibly due to a limited sensitivity of the P-ACCA(S79) antibody. The bulk of these data demonstrate that ACCA phosphorylated at Ser79 interacts with the BRCT domains in vitro as well as with BRCA1 in vivo.

Phosphorylation-dependent Interaction between BRCA1 and ACCA—To determine whether BRCA1 bound both P-ACCA and ACCA or only P-ACCA, GST pull-down assays were performed on MCF7 lysates that had first been treated with APase to dephosphorylate proteins. Western blot analysis using P-ACCA(S79) antibody revealed no P-ACCA in
BRCA1 Modulates Acetyl CoA Carboxylase Activity

![Image of figure 5: BRCT domain binds only the phosphorylated form of ACCA. A, MCF7 cell lysates were treated with or without APPPase, and aliquots of each lysate were analyzed by immunoblotting using P-ACCA(S79) and ACCAL3 antibodies. The lysates were then used in GST pull-down assay. The bound proteins were resolved by immunoblotting using ACCAL3 antibody. Coomassie Blue staining demonstrated that the same amount of GST or GST-BRCT fusion proteins was used in each case. B, GST pull-down assay was performed on MCF7 cells containing increasing amounts of P-ACCA. MCF7 cells were treated as follows: No stress, mechanical lysis in culture dish; AICAR, incubation for 1 h with 0.5 mM of AICAR followed by mechanical lysis in culture dish; Stress, trypsination (conditions used above, in Figs. 4B and 5A). A small amount of lysates was analyzed by immunoblotting using P-ACCA(S79) and ACCAL3 antibodies. Afterward, the lysates were used for GST pull-down assay as above. The bound proteins were resolved by immunoblotting using ACCAL3 antibody. Coomassie Blue staining demonstrated that the same amount of GST or GST-BRCT fusion proteins was used in each case. WB, Western blot.

treated lysates, whereas the total amount of ACCA remained unchanged (Fig. 5A, upper panel). Furthermore, Western blot analysis of the GST pull-down using ACCAL3 antibody that detects both phosphorylated and nonphosphorylated forms of ACCA showed that APPase treatment abolished the interaction between BRCA1 BRCT domains and ACCA (Fig. 5A, lower panel). Because APPase treatment had no effect on the amount of ACCA, these results demonstrate that the BRCA1:ACCA interaction depends on the phosphorylation state of ACCA.

We further investigated whether the amount of BRCA1:ACCA complexes was dependent on the quantity of cellular P-ACCA. It has been shown that several stresses that deplete cellular ATP activate AMPK, leading to phosphorylation and inactivation of ACCA. Similar effects are observed by incubation of cells with AICAR, which causes AMPK activation without affecting intracellular levels of AMP, ADP, or ATP (25). Therefore, three different treatments were applied to MCF7 cells: mechanical harvesting in culture dish, 0.5 mM AICAR incubation for 1 h, or trypsination to induce low, moderate, or hyperphosphorylation of ACCA, respectively. In these conditions, cell lysates showed increasing amounts of P-ACCA without modification in the amount of ACCA (Fig. 5B, upper panel), and the amounts of BRCT-ACCA complexes correlated with the amount of P-ACCA present in the cell lysates (Fig. 5B, lower panel). Therefore, our findings show that the interaction between BRCA1 and ACCA is phosphorylation-dependent and that BRCA1 binds P-ACCA only, in a dose-dependent manner.

Regulation of ACCA Dephosphorylation by BRCA1—Because we determined that BRCA1 binds a phosphorylated and inactive form of ACCA, we further asked whether BRCA1 could modulate ACCA phosphorylation. To test this possibility, we examined whether binding to BRCA1 protects ACCA from dephosphorylation and its subsequent activation. GST pull-down assays were performed on lysates of MCF7 cells previously treated with trypsin to induce a stress, and the BRCT-ACCA complexes were subsequently treated with APPPase. Analysis by Western blotting showed that the amount of ACCA bound to the BRCT domains remained unchanged after APPPase treatment (Fig. 6A). Furthermore, bound ACCA was still phosphorylated as shown by Western blotting analysis. This further indicates that an ACCA region encompassing the Ser79 residue was protected from dephosphorylation in the BRCA1:ACCA complex.

To further investigate whether BRCA1 protects ACCA from dephosphorylation, we examined the effects of BRCA1 silencing on the kinetics of P-ACCA dephosphorylation. In MCF7 cells initially treated with AICAR for 1 h, hyperphosphorylation of P-ACCA was detected (Fig. 6B, 0 min). AICAR was removed at this time (0 min), being the starting point of the time course. Dephosphorylation of P-ACCA initiated 30 min after AICAR removal and was completed after 1 h as shown by Western blotting (Fig. 6B). We verified that AICAR treatment had no effect on the amount of ACCA by immunoblotting using ACCAL3 antibody. To test whether the absence of BRCA1 modulates ACCA dephosphorylation, AICAR treatment was performed on MCF7 cells previously transfected with Si-RNAs. Under these conditions, the kinetics of P-ACCA dephosphorylation in MCF7 cells transfected with Si-control was similar to that of intact MCF7 cells as previously shown in Fig. 6B (Fig. 6C). By contrast, in cells transfected with Si-BRCA1, dephosphorylation of ACCA started immediately and was nearly completed as soon as 10 min after AICAR removal (Fig. 6, C and D). In these cells transfected with Si-RNAs, AICAR treatment had no effect on the amount of ACCA as shown by immunoblotting using ACCAL3 antibody. Furthermore, BRCA1 silencing was not affected by AICAR treatment and during the kinetics of dephosphorylation (Fig. 6C).

These results demonstrate that first BRCA1 protects P-ACCA from dephosphorylation and second BRCA1 silencing results in an acceler-
ated dephosphorylation of ACCA. Therefore, the inhibition of the BRCA1-P-ACCA complex formation renders P-ACCA free to be dephosphorylated.

**BRCA1 Affects Fatty Acid Synthesis through Its Interaction with P-ACCA**—Having demonstrated that in cells treated with AICAR, BRCA1 knockdown rendered P-ACCA free to be dephosphorylated, we further asked whether BRCA1 silencing induced an increase in fatty acid synthesis through its ability to release P-ACCA.

To address this question, MCF7 cells transfected with Si-RNAs were treated with AICAR for 1 h before and for the duration of the 4-h incubation with 2-14C-labeled acetate, and fatty acid synthesis was analyzed. In control cells not treated with AICAR, BRCA1 silencing induced an increase in fatty acid synthesis as first shown in Fig. 1C (Fig. 7, lanes 1 and 2). As anticipated, in cells transfected with Si-control, AICAR treatment largely reduced the lipid synthesis as compared with cells not treated with AICAR (Fig. 7, lanes 1 and 3). However, silencing of BRCA1 in cells treated with AICAR noticeably increased fatty acid synthesis by almost 2-fold (1.76 ± 0.26-fold (mean ± S.E.; n = 3)) as compared with cells transfected with Si-control and treated with AICAR (Fig. 7, lanes 3 and 4).

In these experiments, AICAR was present in cells during all the time of acetate incorporation. This presumably caused ACCA to be rapidly recycled to its phosphorylated state. BRCA1 silencing is expected to
counteract this effect by inducing an accelerated dephosphorylation of ACCA, as observed in Fig. 6D. Therefore, the elevated incorporation of 2-14C-labeled acetate observed in BRCA1 silenced cells probably reflects an increase of fatty acid synthesis in these cells, since ACCA rapidly cycles between its active and inactive states.

**DISCUSSION**

We previously identified an interaction between the tandem of BRCT domains and ACCA that was disrupted by germ line BRCA1 mutations (11). The identification of this novel interaction between BRCA1 and ACCA, the rate-limiting enzyme for the long chain fatty acid synthesis, led us to investigate the biological significance of the BRCA1-ACCA complex.

We decided to examine the potential effect of BRCA1 on lipid synthesis. A number of previous studies have suggested that lipidogenesis is closely linked to tumorigenesis. ACCA is highly expressed in human breast carcinomas (26), and a potential association between the presence of certain ACCA sequence changes and breast cancer development has been recently described (27). Some links between tumorigenesis and FAS, the key metabolic enzyme immediately downstream of ACCA, have also been documented. FAS inhibitors present antitumor effects such as delaying disease progression in a xenograft model of ovarian cancer and delaying mammary tumor development in neu-N transgenic mice (28, 29), and FAS silencing results in growth arrest and cell death (30). FAS is highly expressed in several human malignancies including carcinoma of breast, ovary, endometrium, and prostate (26, 30, 31). In addition, epidemiological studies have associated the development of these cancers with high energy diet and low rate energy expenditure (32). The spectrum of these cancers strongly resembles that of tumors associated with BRCA1 mutations (33). In the present study, silencing of BRCA1, like germ line BRCA1 mutations, abolishes the BRCA1-ACCA interaction. Furthermore, silencing of BRCA1 may reflect the underexpression of BRCA1 commonly observed in sporadic breast and ovarian cancers (34). Therefore, our finding that BRCA1 knockdown can increase lipogenesis is consistent with a physiologic role for BRCA1 in tumorigenesis. In addition, our study further implies that ACCA activity may be regulated by its interaction with BRCA1, thus supporting a model in which control of lipid synthesis would be mediated via the BRCA1-ACCA interaction.

In an attempt to elucidate the molecular mechanism between disruption of the BRCA1-ACCA complex and the increase of lipid synthesis, we deciphered the biochemical characteristics of the BRCA1-ACCA complex. Here, we demonstrated that BRCA1 binds ACCA via its BRCT domains in a phospo-dependent manner. Because AMPK treatment induces a global dephosphorylation of cellular proteins, the GST pull-down experiments performed on AMPK-pretreated lysates demonstrated that BRCA1 binds a phosphorylated form of ACCA that remained to be defined. AICAR induces ACCA phosphorylation at Ser/Thr via AMPK and the antibody P-ACCA(S79) used here is specific to phosphorylated Ser/Thr. Therefore, the bulk of our experiments indicate that an ACCA region phosphorylated at least at Ser/Thr was protected from dephosphorylation in the BRCA1-ACCA complex. Because ACCA is inactivated when phosphorylated on Ser/Thr (14, 35), our results clearly demonstrated that BRCA1 binds the inactive form of ACCA. Further studies will be needed to delineate the entire phosphorylated region of ACCA implicated in this interaction.

BRCT domains, originally proposed to mediate protein-protein interactions (7), have recently been described as phosphoprotein-binding domains (8–10). Our findings fully support this recent notion. Here we provide evidence for a new signaling function of BRCT domains, influencing the biological process of lipogenesis. BRCA1 acts as a scaffold protein implicated in multiple cellular functions, such as transcription, DNA repair, and ubiquitination (36). In this regard, it is noteworthy that the implication of BRCA1 in these functions is greatly linked to the functions of the many BRCA1-interacting proteins identified to date (3, 37). In our study, the potential of BRCA1 to affect lipogenesis in mammary epithelial cells is strongly associated with its P-ACCA binding property, providing a new mechanism by which BRCA1 may exert tumor suppressor functions.

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