Analysis of Murine Brca2 Reveals Conservation of Protein-Protein Interactions but Differences in Nuclear Localization Signals*

Christopher J. Sarkisian†, Stephen R. Master‡, L. Julie Huber†, Seung I. Ha† and Lewis A. Chodosh‡§¶

From the ‡Department of Molecular and Cellular Engineering and §Division of Endocrinology, Diabetes and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6160

In this report, we have analyzed the protein encoded by the murine Brca2 locus. We find that murine Brca2 shares multiple properties with human BRCA2 including its regulation during the cell cycle, localization to nuclear foci, and interaction with Brca1 and Rad51. Murine Brca2 stably interacts with human BRCA1, and the amino terminus of Brca2 is sufficient for this interaction. Exon 11 of murine Brca2 is required for its stable association with RAD51, whereas the carboxyl terminus of Brca2 is dispensable for this interaction. Finally, in contrast to human BRCA2, we demonstrate that carboxyl-terminal truncations of murine Brca2 localize to the nucleus. This finding may explain the apparent inconsistency between the cytoplasmic localization of carboxyl-terminal truncations of human BRCA2 and the hypomorphic phenotype of mice homozygous for similar carboxyl-terminal truncating mutations.

Women inheriting mutations in the BRCA2 tumor-suppressor gene have up to an 84% lifetime risk of developing breast cancer (1), and these tumors account for ~35% of inherited breast cancers in women (2). BRCA2 encodes a 3418-amino acid nuclear protein of a predicted molecular mass of 384 kDa. Most disease-causing BRCA2 alleles contain truncating mutations that result in deletion of the three characterized nuclear localization signals present at the extreme carboxyl terminus of BRCA2 (3, 4). Because these signals are required for the nuclear localization of human BRCA2, it has been postulated that truncating alleles of BRCA2 are functionally equivalent to null alleles of this tumor suppressor gene (3).

Though its exact cellular role remains unclear, a growing body of evidence indicates that BRCA2 is involved in DNA damage-response pathways shared with BRCA1 and RAD51. BRCA2, BRCA1, and RAD51 are each co-regulated with highest levels of expression occurring during the S and G2/M phases of the cell cycle, and these proteins co-localize to discrete foci within the nucleus (5–11). Furthermore, human BRCA2 has been shown to physically interact with both RAD51 (12–16) and BRCA1 (16).

Human BRCA2 binds to RAD51 via eight BRC repeats, each 30–80 amino acids in length, that are located within exon 11 of BRCA2 (17, 18). These repeats have been demonstrated by yeast two-hybrid analysis to be both necessary and sufficient for stable binding of human BRCA2 to RAD51 (12, 14, 15). The region(s) of BRCA2 that are required for binding to BRCA1 have been less clearly defined, though the carboxyl-terminal third of BRCA2 has been shown to be dispensable for this interaction (16). Nevertheless, despite the identification of BRCA2-RAD51 and BRCA2-BRCA1 protein-protein interactions, the contribution of these interactions to the tumor-suppressor functions of BRCA2 remains uncertain.

Mice bearing homozygous mutations in Brca2 that yield truncations of all eight BRC repeats uniformly die in utero between embryonic day 6.5-8.5, with elevated levels of p53 and p21 (19–21). Notably, this phenotype is similar to that of mice homozygous for null mutations in either Rad51 or Brca1 (22–26). Whereas mice bearing truncating alleles of Brca2 that remove only a subset of BRC repeats also die in utero, a fraction of homozygotes survive to birth with the survival rate being roughly proportional to the number of BRC repeats left intact (27–29). Surviving homozygotes invariably succumb to thymic lymphomas, and cells from these mice exhibit increased genotoxic sensitivity and chromosomal instability, as well as an impaired ability to form Rad51 nuclear foci in response to DNA damage (27–30). In contrast, mice homozygous for truncating mutations in Brca2 that leave exon 11 intact exhibit a more limited sensitivity to genotoxins, are 100% viable, and do not appear to develop spontaneous tumors (31). These data argue for a central role of exon 11 in the genomic surveillance and tumor-suppressor functions of Brca2.

Whereas murine knockout models support a role for BRCA2 as a tumor suppressor, the increasingly severe defects observed in mice as larger amounts of the Brca2 carboxyl terminus are truncated appear inconsistent with reports that even small carboxyl-terminal truncations in human BRCA2 result in its cytoplasmic localization. That is, essentially all truncating alleles might be expected to behave similarly to null alleles, because carboxyl-terminal truncation would ostensibly lead to cytoplasmic localization and preclude Brca2 from participating in nuclear functions (3, 4). This apparent discrepancy could be because of differences in the subcellular localization signals of human and murine Brca2 or to differences in the functions of murine and human BRCA2 in the cytoplasm. In this regard, another apparent functional difference between murine and human BRCA2 is suggested by the mapping of a murine Brca2-Rad51 interaction to the carboxyl terminus of murine Brca2, because similar approaches have shown that the corresponding region of human BRCA2 lacks significant affinity for RAD51 (12, 14, 15). Further complicating the direct comparison of
 murine and human BRCA2 is the fact that the overall amino acid homology between these orthologs is only 59%, a relatively low degree of evolutionary conservation compared with other tumor-suppressor genes. Together, these data have called into question the applicability of murine models for understanding the function of human BRCA2.

In this report, we characterize the murine Brca2 protein. We find that Brca2 stably interacts with murine Brca1 and Rad51. We demonstrate that the physical association of Brca2 with Rad51 requires exon 11 of murine Brca2 but not its carboxyl terminus. We also show that murine Brca2 differs from human BRCA2 in that carboxyl-terminal truncations of murine Brca2 localize to the nucleus. Collectively, our findings suggest that multiple functional interactions of Brca2 have been evolutionarily conserved with the notable exception of those signals required for its nuclear localization.

**EXPERIMENTAL PROCEDURES**

*Isolement of Murine Brca2 cDNA—Poly(A)*

- RNA isolated from day 14 murine embryos was used to generate a cDNA library in lambda ZAP using the ZAP-cDNA synthesis and ZAP-cDNA Gigapack II Gold packaging kits according to the manufacturer's instructions (Stratagene). 5 × 10^6 plaques from each library were screened by standard methods using [32P]dCTP-labeled random-primer cDNA fragments (BMB) corresponding to nucleotides 2–221, 798–2932, and 9033–9972 of murine Brca2. Hybridization was performed at a concentration of 10^6 cpm/ml in 48% formamide, 10% dextran sulfate, 4.8 × SSC, 20 ml Tris, pH 7.5, 10 × Denhardt's solution, 20 μg/ml salmon sperm DNA, and 0.1% SDS at 42 °C overnight. Filters were washed twice in 2 × SSC/0.1% SDS at room temperature for 20 min and twice in 0.2 × SSC/0.1% SDS for 20 min at 50 °C and subjected to autoradiography on XAR-5 film (Eastman Kodak Co.). Phage clones were plaque purified, and plasmids were liberated by *in vivo* excision according to the manufacturer's instructions. Sequence analysis identified three overlapping clones that together spanned the entire coding sequence, with the exception of an internal deletion of nucleotides 454–672. This region was replaced with a polymerase chain reaction product generated from murine testis first-strand cDNA and primers 5′-GAATTCATGCCCGTTGAATA-3′ and 5′-CATCCGAGGAATTCCTGCAG-3′. After sequencing to verify the absence of additional mutations, the overlapping clones were assembled to generate a full-length murine Brca2 cDNA.

*Generation of Antisera—Using primers 5′-CATCCGAGGAATTCCTGCAG-CACAGCGATTTAGGAC-3′ and 5′-CATCCCGAGCAGCTTCTTCATCTTGGC-3′. After sequencing to verify the absence of additional mutations, the overlapping clones were assembled to generate a full-length murine Brca2 cDNA.*

**Cell Cycle Synchronization and Analysis**—HC11 cells were synchronized by serum starvation for 48 h and were subsequently restimulated for 20 h in medium containing 20% serum. Cells were trypsinized and washed in PBS, and approximately two-thirds of cells were pelleted and snap-frozen for subsequent protein harvest. The remaining cells were pelleted, resuspended in PBS, and fixed in 70% ethanol. Following fixation, cells were pelleted, resuspended in PBS supplemented with 10 μg/ml propidium iodide and 100 μg/ml RNase A, and sorted by DNA content using a Becton Dickinson FACScan flow cytometer. The program ModFit was used to quantify percentages of cells in each phase of the cell cycle.

**Subcellular Fractionation**—Nuclear and cytoplasmic fractionation was performed as described previously (34). Briefly, 16MB9A cells were harvested by trypsinization, pelleted, and washed in PBS. Cells were washed in ice-cold hypertonic buffer (30 mM HEPES, pH 7.5, 5 mM KCl, 1 mM MgCl₂, resuspended in three packed cellular volumes of hypertonic buffer supplemented with protease inhibitors, and incubated on ice for 30 min. Cells were homogenized in a Wheaton Dounce with 25 strokes of a type B pestle. An equal volume of Nonidet P-40 (0.1% Igepal CA-630, 250 mM sucrose, 10 mM Tris, pH 7.5) was added dropwise, and cells were lysed using another 10 strokes. Nuclei were pelleted at 1300 × g for 4°C for 5 min. Following removal of the cytoplasmic supernatant, nuclei were washed twice in 1.1 hypotonic buffer/Nonidet P-40 lysate buffer and resuspended in an amount of 1:1 hypotonic buffer/Nonidet P-40 lysate buffer equal to the extract volume prior to centrifugation of nuclei. Nuclear and cytoplasmic fractions were diluted with 6× EBC to a final concentration of 1×, centrifuged to remove insoluble debris, and boiled in 1× (final) Laemmli sample buffer prior to SDS-PAGE.

**Immunofluorescence**—Cells were cultured in 2-well culture slides (Falcon), rinsed in PBS, and fixed for 10 min in 3% paraformaldehyde/2% sucrose/PBS. Cells were rinsed twice in PBS and permeabilized for 5 min in ice-cold buffer (0.5% Triton, 20 mM HEPES, pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose). Following five rinses in PBS, cells were incubated at 37 °C for 20 min with anti-Brc2A (2 μg/ml in 3% bovine serum albumin/PBS). Cells were rinsed twice in PBS and air-dried before staining.
stained with a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) in 3% bovine serum albumin/PBS. Stained cells were rinsed three times in PBS, mounted in Vectastain (Vector Laboratories), and visualized using a Bio-Rad MRC-1024 confocal microscope with a Kr/Ar laser. For subcellular localization of Brca2 isoforms, tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit (Jackson Immunoresearch) was used as a secondary antibody at a 1:200 dilution, and cells were visualized using confocal microscopy as above.

RESULTS

Generation of Antisera to Murine Brca2—To investigate the function of the murine Brca2 protein, we raised antisera to two recombinant Brca2 polypeptides corresponding to amino acid residues 19–135 and 206–566. Sera from these rabbits were affinity-purified and designated anti-Brca2A and anti-Brca2B, respectively. To demonstrate that these antibodies specifically recognize murine Brca2, we transfected 293T cells with either a full-length cDNA encoding Brca2 or an empty vector control. Immunoblotting of whole cell extracts with anti-Brca2A demonstrated a band of the predicted molecular mass in 293T cells transfected with a Brca2 cDNA but not in cells transfected with a control vector (Fig. 1A). This band co-migrates with endogenous Brca2 detected in the mammary epithelial cell lines HC11, NMuMG, and 16MB9A. Endogenous human BRCA2, detected in 293T cells using an antibody recognizing a carboxyl-terminal epitope of BRCA2, also co-migrates with this band (Fig. 1A). In contrast, no co-migrating band is detected by immunoblot of whole cell extracts from CAPAN-1 cells, in which a 6174dT frameshift mutation results in a carboxyl-terminal truncation of the BRCA2 gene product (Fig. 1A). Detection of murine Brca2 was completely blocked by pre-incubating anti-Brca2A with the cognate GST-Brca2 (residues 19–135) fusion protein but not by pre-incubating with GST (data not shown). These findings indicate that anti-Brca2A specifically detects murine Brca2.

As further evidence of the specificity of anti-Brca2A, we performed reciprocal immunoprecipitations and immunoblots on murine mammary epithelial cell extracts with anti-Brca2A and anti-Brca2B. As shown in Fig. 1B, anti-Brca2A detects a polypeptide of the appropriate molecular mass present in cell extracts subjected to immunoprecipitation with anti-Brca2B but not with control rabbit IgG. Similarly, anti-Brca2B detects a polypeptide of the appropriate molecular mass in cell extracts subjected to immunoprecipitation with anti-Brca2A. Moreover, the polypeptide immunoprecipitated by anti-Brca2A and anti-Brca2B co-migrates with the polypeptides detected by these antibodies in whole cell extracts (see below, and see Fig. 4). In aggregate, these data demonstrate that anti-Brca2A and anti-Brca2B specifically recognize murine Brca2.

Brca2 Protein Levels Are Up-regulated during S and G2/M Phases of the Cell Cycle—Our laboratory has demonstrated previously that steady-state levels of murine Brca2 mRNA are regulated during the cell cycle with peak expression occurring near the G1/S transition (35). Human BRCA2 has been shown to exhibit a similar pattern of regulation at both the RNA and protein levels (5, 6). To determine whether steady-state levels of murine Brca2 protein are similarly regulated, HC11 cells were synchronized by serum starvation for 48 h, restimulated with serum, and harvested at 4-h intervals. Immunoblotting revealed that murine Brca2 expression is up-regulated beginning ~12 h following restimulation with serum, which coincides with cellular entry into S phase as shown by flow cytometry and up-regulation of cyclin A (Fig. 2). This up-regulation persists as cells exit S phase and enter the G2/M phase of the cell cycle (Fig. 2). Similar results were observed in a second mammary epithelial cell line, NMuMG (data not shown). These findings indicate that the cell cycle regulation of BRCA2 is conserved between mouse and human at both the RNA and protein level.

Murine Brca2 Localizes to Nuclear Foci—Human BRCA2 has been shown to localize to nuclei by biochemical subcellular fractionation (6). To determine whether murine Brca2 shares this property, nuclear and cytoplasmic fractions were prepared from the murine mammary epithelial cell line, 16MB9A. The purity of these fractions was confirmed by immunoblotting for β-tubulin and RAD50 as controls for cytoplasmic and nuclear proteins, respectively (Fig. 3A). This analysis demonstrated that murine Brca2 is found primarily in the nuclear fraction of 16MB9A cells (Fig. 3A).

Consistent with the nuclear localization of human BRCA2, immunofluorescence studies have shown that this protein localizes to subnuclear foci (16). To determine whether murine Brca2 exhibits a similar localization, we performed indirect
immunofluorescence on NMuMG cells using anti-Brca2A. Numerous nuclear foci were observed in cells stained with anti-Brca2A but not in cells stained with a secondary antibody alone (Fig. 3B). Fluorescent signal was completely blocked by the cognate GST-Brca2 (residues 19–135) fusion protein but not by GST alone (data not shown). Collectively, these data demonstrate that, similar to human BRCA2, murine Brca2 is a nuclear protein and localizes to discrete foci in mammary epithelial cell lines.

**Brca2 Stably Interacts with Rad51 and Brca1**—Human BRCA2 has been demonstrated previously to physically interact with the homology-based DNA repair protein, RAD51 (12–16). This interaction has been shown to be direct by both yeast two-hybrid and in vitro GST pulldown assays (12, 14, 15). Although murine Brca2-GST fusion proteins have been shown to bind to Rad51 in vitro, and murine Brca2-GAL4 fusion proteins have been shown to bind to Rad51 by yeast two-hybrid approaches (19, 32), endogenous murine Brca2 and Rad51 have yet to be shown to interact in mammary epithelial cells in vivo. To address this, we performed reciprocal co-immunoprecipitations of Rad51 and Brca2 from HC11 cells (Fig. 4A). This analysis demonstrated that Rad51 antisera were equally as effective as Brca2 antibodies in immunoprecipitating Brca2, suggesting that the majority of Brca2 in these cells is stably bound to Rad51. In contrast, Brca2 antisera co-immunoprecipitated only a fraction of the total Rad51 present in these cells (Fig. 4A). Consistent with this finding, a similarly small proportion of RAD51 has been found complexed with BRCA2 in human cells (13, 16). Together, these observations suggest that within cells there is a large pool of Rad51 molecules that are not complexed with Brca2. Although we cannot rule out the possibility that there is a significant pool of Brca2 molecules that are not immunoprecipitated by our Brca2 antibodies, this latter explanation appears unlikely given that anti-Brca2B antibodies immunoprecipitate the majority of Brca2 present in these cells as judged by comparison to input material (Fig. 4A).

Because human BRCA2 has also been demonstrated to interact with BRCA1 (16), we wished to determine whether an analogous interaction exists in murine cells. Using polyclonal antibodies raised against murine Brca1 residues 69–278 (mAb-1) and residues 995–1244 (mAb-3) (36), we performed reciprocal co-immunoprecipitations of Brca1 with Brca2. Low
but significant levels of Brca2 were detected in Brca1 immunoprecipitates (Fig. 4B). Based on comparisons to input cellular extracts, less than 5% of total Brca2 polypeptides appear to be stably bound to Brca1 under these conditions (Fig. 4B). This finding is consistent with the fraction of total BRCA2 molecules that have been reported to be bound to BRCA1 in human cells (16). Conversely, we detected Brca1 in Brca2 immunoprecipitates at levels approximately equivalent to those present in Rad51 precipitates (Fig. 4B). No Brca1 or Brca2 was detected in rabbit IgG control precipitates. Taken together, our data indicate that the proteins with which murine Brca2 interacts are similar to those with which human BRCA2 interacted and that the stoichiometries with which these interactions occur may also be similar. Of note, however, we cannot rule out the possibility that our antibodies have incomplete access to cellular Brca2-Brca1 complexes or that the immunoprecipitation conditions employed in these experiments cause disruption of Brca2-Brca1 complexes.

**Exon 11 of Murine Brca2, but Not Its Carboxyl Terminal, Is Required for Interaction with Rad51**—The domain(s) with which murine and human BRCA2 each interact with RAD51 have been noted previously to differ (37). Specifically, murine Brca2 has been shown to interact with Rad51 via its carboxyl terminus by yeast two-hybrid and *in vitro* GST pulldown assays (19, 38). However, despite the high degree of evolutionary conservation of this domain, similar approaches have indicated that the corresponding domain of human BRCA2 does not stably interact with Rad51 (12, 14, 15). Rather, the BRC repeats of human BRCA2 have been shown to be necessary and sufficient for interaction with RAD51. To date, the contribution of the BRC repeats of murine Brca2 to its interaction with Rad51 have not been addressed. We generated several deletion mutants of Brca2 to identify those domain(s) required for Rad51 interaction (Fig. 5A). The expression construct, *Brca2*Δ*Bst*, contains an in-frame deletion of the majority of exon 11 that spans all 8 BRC repeats. *Brca2ΔC* contains a deletion of all *Brca2* sequences 3' of exon 11, including the reported carboxy-terminal Rad51 interaction domain (19, 38). Finally, *Brca2*Δ*Bst-C* deletes both exon 11 and carboxy-terminal sequences. Each of these constructs retains the epitopes recognized by anti-Brca2A and anti-Brca2B.

293T cells were transfected with expression constructs for full-length *Brca2*, *Brca2* deletion mutants, or a *pBKCMV* control vector, and harvested cell extracts were subjected to immunoprecipitation for Brca2 and RAD51. This analysis revealed that full-length Brca2 and Brca2ΔC were comparable in their ability to co-precipitate RAD51 (Fig. 5B). In contrast, no RAD51 was detected in anti-Brca2 immunoprecipitates from cells transfected with *Brca2*Δ*Bst* or *Brca2ΔBst-C*, despite the fact that these Brca2 mutant polypeptides were expressed at levels that exceeded those of full-length Brca2 and Brca2ΔC (Fig. 5B). Performing the reciprocal co-immunoprecipitation experiment in 293T cells yielded similar results, as full-length Brca2 and Brca2ΔC were found to co-immunoprecipitate with RAD51, whereas Brca2ΔBst and Brca2ΔBst-C failed to co-precipitate with RAD51 (Fig. 5C). These results were also observed when Brca2ΔBst and Brca2ΔBst-C were expressed at levels comparable with those of full-length Brca2 and Brca2ΔC (data not shown) indicating that the failure of Brca2ΔBst and Brca2ΔBst-C to interact with RAD51 is not an artifact of their higher expression levels. Moreover, the fact that human and murine RAD51 are identical at the amino acid level, and that the BRCA2-RAD51 interaction has been shown to be direct, suggests that the inability of Brca2ΔBst and Brca2ΔBst-C to co-precipitate RAD51 is not due to differences in RAD51 sequences or bridging molecules present in human cells (12, 14, 15). Collectively, these data strongly suggest that exon 11 is the principal RAD51 interaction domain contained within murine Brca2.

**The Amino Terminal of Murine Brca2 Is Sufficient for Interaction with Human BRCA1**—Brca1 and Brca2 each display relatively low overall similarity to their human orthologs (~59%). Given this, we wished to define the domain(s) of Brca2 required for interaction with Brca1 and to determine whether the Brca2-Brca1 interaction would be preserved when one of these proteins was replaced by its human counterpart. Extracts of 293T cells transfected with full-length *Brca2* or *Brca2* deletion constructs, along with a *BRCA1* expression construct, were subjected to Brca2 immunoprecipitation and immunoblotted for co-precipitating BRCA1 (Fig. 6). This analysis revealed that each of the mutant Brca2 isoforms tested were comparable in their ability to interact with human BRCA1. Similar results were observed in co-immunoprecipitations performed using anti-Brca2A or anti-Brca2B and in the absence of ectopic BRCA1 expression (data not shown). Low levels of BRCA1 were detectable in anti-Brca2A precipitates from 293T cells transfected...
with the control vector, *pBKCMV*, suggesting that anti-Brca2A may cross-react, albeit weakly, with human BRCA2 (Fig. 6). In aggregate, our findings suggest that sequences within the amino-terminal 738 residues of Brca2 are sufficient for interaction with BRCA1, although additional domains of Brca2 may contribute to the stability of this association. These findings are consistent with experiments demonstrating that the interaction of human BRCA2 and BRCA1 is preserved in CAPAN-1 cells, which express a BRCA2 protein that lacks the carboxyl-terminal third of BRCA2 (16). To date, the lack of an antibody to the carboxyl terminus of Brca2 and inefficient expression of epitope-tagged Brca2 deletion mutants (data not shown) have prevented us from testing whether the amino terminus of Brca2 is required for its interaction with BRCA1. Nevertheless, we conclude that the amino terminus of murine Brca2 is sufficient to stably interact with human BRCA1 and that the se-

**Fig. 5.** Exon 11, but not the carboxyl terminus, of murine Brca2 is required for interaction with RAD51. **A**, schematic depicting deletion mutants of *Brca2* generated from a full-length *Brca2* cDNA. *Brca2ΔBst* represents an internal deletion mutant that encodes a polypeptide lacking amino acid residues 738–2278 from exon 11. *Brca2ΔC* lacks sequence from the second *Bst*EII site, at residue 2280, to the carboxyl terminus of the protein, and *Brca2ΔBst-C* lacks sequence from the first *Bst*EII site, at residue 742, to the carboxyl terminus of the protein. **B**, 293T cells were transfected with 25 μg of the indicated *Brca2* constructs. Cellular extracts (**left panels**) or cellular extracts immunoprecipitated (**IP**) with anti-Brca2B (**right panels**), were separated by SDS-PAGE and immunoblotted with either anti-Brca2A (**top panel**) or anti-RAD51 Ab-1 (NeoMarkers; **bottom panel**). **C**, 293T cells were transfected with 25 μg of the indicated *Brca2* constructs. Whole cell extracts (**left panels**) or whole cell extracts immunoprecipitated with anti-RAD51 Ab-1 (Oncogene Science; **right panels**) were immunoblotted (**IB**) with anti-Brca2A. The **bottom panels** were separated by 15 rather than 5% SDS-PAGE to enhance the resolution of *Brca2ΔBst-C*. 
FIG. 6. The amino terminus of Brca2 is sufficient for interaction with BRCA1. 293T cells were transfected with 12.5 μg of pBKCMV, 12.5 μg of full-length Brca2, 2 μg of Brca2ΔBst, 12.5 μg of Brca2ΔAC, or 2 μg of Brca2ΔBst-C. All transfections included 12.5 μg of a BRCA1 expression plasmid and sufficient pBKCMV to bring the total DNA to 25 μg per transfection. Whole cell extracts (left panel) or anti-Brca2A immunoprecipitates (right panels) from transfected cells were immunoblotted (IB) with anti-BRCA1 MS110 (top panels) or anti-Brca2A (bottom panel). IP, immunoprecipitated.

As expected, exogenously expressed full-length murine Brca2 was observed to localize exclusively to the nucleus, as demonstrated by co-fluorescence with ECFP-Nuc (CLONTECH), a control for nuclear localization (Fig. 7). Similarly, Brca2ΔBst was also shown to localize to the nucleus of transfected cells, indicating that exon 11 is not required for the nuclear localization of murine Brca2. Surprisingly, Brca2ΔBst-C was also shown to localize to the nucleus despite its deletion of more than three-fourths of the full-length protein, including the carboxyl terminus. Brca2ΔBst-C encodes a polypeptide with a predicted molecular mass of 82 kDa that is significantly greater than the 65-kDa molecular mass cutoff for passive diffusion through nuclear pores (39). As such, the nuclear localization of Brca2ΔBst-C cannot be explained by simple diffusion. Moreover, no fluorescent signal was detected in control cells transfected with the empty vector, indicating that the apparent localization of Brca2ΔBst-C is not the result of antibody cross-reactivity with endogenous human BRCA2. These findings suggest that the amino terminus of murine Brca2 is sufficient to direct the nuclear localization of this protein.

DISCUSSION

We have demonstrated that the murine Brca2 protein is similar to human BRCA2 with regard to its nuclear localization, cell cycle regulation, binding to Brca1, and binding to Rad51. In addition, we have defined further the domains of Brca2 that are required for its interaction with RAD51 and BRCA1. Finally, despite low overall homologies between the murine and human orthologs of BRCA2 and BRCA1, we have demonstrated that murine Brca2 is capable of stably interacting with human BRCA1 in vivo. This indicates that the interaction between Brca1 and Brca2 has been conserved evolutionarily and suggests that this interaction is functionally important. In aggregate, our data are consistent with the hypothesis that murine and human BRCA2 have largely equivalent functions.

One notable difference that we observed between murine and human BRCA2 was the finding that the amino terminus of murine Brca2 appears to be sufficient for its nuclear localization. In contrast, analysis of human BRCA2-GFP fusion proteins has demonstrated that truncating even 155 residues from the carboxyl terminus of BRCA2 completely abrogates its nuclear localization (3). Similarly, endogenous BRCA2 in CAPAN-1 cells, which lacks the carboxyl-terminal third of BRCA2, has been shown to localize to the cytoplasm by biochemical fractionation (3). As such, we believe that our findings reflect differences in the placement of nuclear localization sequences within human and murine Brca2. A potential caveat to this interpretation is that our localization studies were performed on Brca2 polypeptides expressed ectopically in human 293T cells rather than in murine cells. However, our conclusions are supported by the finding that a targeted deletion of the final 566 coding nucleotides of Brca2 results in a polypeptide that localizes to the nucleus in murine cells (40). Paradoxically, the difference in positioning of nuclear localization signals that we have identified between murine and human BRCA2 strengthens the hypothesis that murine and human BRCA2 are functionally equivalent. If, as for human BRCA2, truncation at the
extreme carboxyl terminus of murine Brca2 resulted in cytoplasmic localization, we would have been forced to conclude that the more severe phenotype of mice bearing amino-terminal compared with carboxyl-terminal truncating Brca2 mutations reflected differences in the functions of murine and human BRCA2. Our demonstration that carboxyl-terminal truncations of murine Brca2 remain nuclear resolves this dilemma.

Mice homozygous for truncating mutations within exon 11 exhibit reduced embryonic survival, spontaneous tumorigenesis, genomic instability, and reduced Rad51 nuclear foci formation following DNA damage (27–30). Our finding that Brca2 polypeptides lacking exon 11 are incapable of co-immunoprecipitating RAD51 constitutes the first biochemical evidence that exon 11 is required for the interaction of Brca2 with RAD51. This, along with our observation that Brca2 mutants lacking the carboxyl terminus retain their capacity to bind to RAD51, is consistent with yeast two-hybrid studies of the human BRCA2-RAD51 interaction and suggests that exon 11 is the principal domain of murine Brca2 required for binding to RAD51. Nevertheless, given that interactions of the carboxyl terminus of murine Brca2 with Rad51 have been detected by two different approaches, we favor the possibility that this region may contribute to the interaction of Brca2 with Rad51.

The multiple similarities between human and murine Brca2 that we have demonstrated in this report shed new light on observations made in mice bearing targeted mutations in Brca2. Mice lacking the small carboxyl-terminal domain of Brca2 shown to interact with Rad51 have not been reported to develop tumors, although cells from such mice exhibit premature senescence and decreased efficiency in homology-based DNA repair (31, 40). Our data predict that mice bearing carboxyl-terminal deletions would have at most only slightly impaired binding of Brca2 to Rad51. This, in turn, may explain the more modest phenotype of mice bearing such mutations. In support of this hypothesis, Moynahan and colleagues (40) have shown recently that the amount of Brca2 bound to Rad51 in murine embryonic stem cells is unaffected by deletion of the carboxyl-terminal domain of Brca2. As such, the premature senescence and decreased DNA repair phenotypes observed in these mice may be due either to an uncharacterized defect in the Rad51 pathway or to the disruption of interactions with other proteins involved in homology-based DNA repair.

Finally, our finding that the amino terminus of murine Brca2 is sufficient to interact with BRCA1 suggests that Brca2-Brca1 complexes may be maintained in all Brca2 knockout mouse models generated to date; however, whether such Brca2-Brca1 complexes retain their function is unknown. Both Brca2 and Brca1 mutant cells have defects in Rad51 focus formation following DNA damage (36, 41). We have recently demonstrated that murine Brca1, like murine Brca2, localizes to nuclear foci (36). As peptides bearing consensus BRC repeat sequences can inhibit the polymerization of RAD51 onto DNA substrates in vitro (42), these data collectively suggest a role for Brca2, and potentially Brca1, in recruiting or preparing Rad51 for subsequent recombination events at sites of DNA damage. Nevertheless, it has yet to be demonstrated how Brca2 and Brca1 orchestrate Rad51 nuclear focus formation following DNA damage, and it has not been determined whether the disrupted regulation of Rad51 nuclear focus formation is ultimately responsible for the malignant transformation of Brca1 and Brca2 mutant cells. Such studies should enhance our understanding of the mechanism by which Brca1 and Brca2 gene products suppress tumor formation.

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