The Nuclear Localization Sequences of the BRCA1 Protein Interact with the Importin-α Subunit of the Nuclear Transport Signal Receptor*

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The BRCA1 gene product is a nuclear phosphoprotein that is aberrantly localized in the cytoplasm of most breast cancer cells. In an attempt to elucidate the potential mechanism for the nuclear transport of BRCA1 protein, three regions of highly charged, basic residues, 503KKRRRP508, 606PKKNLRKKS615, and 651KKKYN656, were identified as potential nuclear localization signals (NLSs). These three regions were subsequently mutated to 503KL508, 607KLS613, and 653KLN656, respectively. Wild-type and mutated proteins were tagged with the flag epitope, expressed in human DU145 cells, and detected with the M2 monoclonal antibody. In DU145 cells, the KLP protein mutant completely fails to localize in nuclei, whereas the KLS mutant is mostly cytoplasmic with occasional nuclear localization. The KLN protein is always located in nuclei. Consistently, hSRP1α (importin-α), a component of the NLS receptor complex, was identified in a yeast two-hybrid screen using BRCA1 as the bait. The specificity of the interaction between BRCA1 and importin-α was further demonstrated by showing that the 503KKRRRP508 and 606PKKNLRKKS615 regions, but not 651KKKYN656, are critical for this interaction. To determine if the cytoplasmic mislocation of endogenous BRCA1 in breast cancer cells is due to a deficiency of the cells, wild-type BRCA1 protein tagged with the flag epitope was ectopically expressed in six breast cancer cell lines. The analysis demonstrated that, in all six, this protein localized in the cytoplasm of these cells. In contrast, expression of the construct in four non-breast cancer cell lines resulted in nuclear localization. These data support the possibility that the mislocation of the BRCA1 protein in breast cancer cells may be due to a defect in the cellular machinery involved in the NLS receptor-mediated pathway of nuclear import.

BRCA1, located on chromosome 17q21, was cloned and shown to be responsible for about 50% of familial breast and ovarian cancers (1). The protein encoded by this gene contains a zinc finger motif and an acidic block of residues (1). These features suggest that BRCA1 may function as a transcription factor, although there is no experimental evidence so far to support this supposition. Similarly, the precise biological function of BRCA1 protein remains unclear. There are several lines of circumstantial evidence, however, suggesting that BRCA1 may have a role in cellular growth and differentiation. First, BRCA1 mRNA is highly expressed in tissues where cells are rapidly proliferating and differentiating (2, 3). Second, homozygous deletion of the BRCA1 gene in mice causes lethality in early embryogenesis due to the retardation of cell growth and malformation of the embryo (4–6). Third, the expression of both BRCA1 mRNA and protein is increased following cell cycle progression. Moreover, phosphorylation of the BRCA1 protein by cyclin-dependent kinases is also positively regulated during the cell cycle (7).

As a tumor suppressor gene, it is unusual that mutations in BRCA1 are closely linked to inherited breast and ovarian cancers but are rarely found in sporadic tumors (1, 8, 9). This result has raised questions concerning the authenticity of the BRCA1 gene as a breast tumor suppressor (10). However, inactivation of tumor suppressor proteins can be independent of their genetic mutations. For example, wild-type p53 protein has also been found mislocalized in the cytoplasm of breast cancer cells, while mutant p53 remains in the nucleus (11). These studies suggested the existence of multiple pathways for the inactivation of p53 function in breast cancer cells. It is therefore possible that BRCA1 may be functionally inactivated by its mislocation from the nuclear to cytoplasmic compartment in sporadic breast cancer cells.

Nuclear transport is a complicated process involving multiple factors. Since it is a large molecule, it is likely that the BRCA1 protein is actively translocated from the cytoplasm to the nucleus by the NLS receptor-mediated transport system (reviewed in Refs. 12 and 13). The direct import of karyophilic proteins through the nuclear pore complex requires energy (14, 15) and an NLS located in the transport substrate (16, 17) to which a cytosolic receptor complex, importin-α and importin-β, binds (18, 19). A GTP-binding protein, Ran, mediates the energy-dependent translocation of the substrate-receptor complex through the nuclear pore complex (20). After translocation, importin-β dissociates from the complex in the vicinity of the inner aspect of the nuclear envelope while importin-α accompanies the substrate to its sites of function (21). Any defect in this transportation system could lead to a failure in the translocation of BRCA1 to the nucleus. However, it is very unlikely that the cells could survive a major deficit in nuclear transport.

In contradiction to the findings of ourselves (22, 23) and others (24), it has been reported that BRCA1 is a secreted protein (25). Since the subcellular location of proteins is a fundamental aspect of their function, it is important to solidify

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1 The abbreviation used is: NLS, nuclear localization signal.
the data regarding the location of BRCA1 in normal and cancer cells. In an attempt to address these questions, we initiated experiments to investigate the nuclear transport of the BRCA1 protein by ascertaining the identity of its functional NLS motifs, by identifying proteins with which it interacts, and by extending the investigation of its subcellular distribution in breast cancer cells. Our results indicate that there are two functional nuclear localization sequences in the BRCA1 protein that interact with hSRP1α (importin-α or karyopherin-α (18, 26, 27)). Furthermore, ectopically expressed wild-type BRCA1 protein is located in nuclei of normal and non-breast tumor cell lines but is detected in the cytoplasm of all breast cancer cell lines tested.

**Experimental Procedures**

**Cell Culture and DNA Transfections—**Human cell lines DU145 (prostate cancer), T24 (bladder cancer), T47D, ZR75, MB231, MB468, MDA330, MCF7 (breast cancer), HBL100 (normal breast epithelial cells immortalized with SV40), and CV1 (monkey kidney cell line) were grown at 37°C in a humidified 10% CO2-containing atmosphere in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Inc.) on plastic surfaces. Each 10-cm dish of cells grown to 60% confluency was transfected with 10 μg of plasmid DNA using the calcium phosphate method (28). The calcium phosphate precipitate was left at room temperature for 6–8 h. At that time the medium was drained, and the cells were refed with fresh medium.

**NLS Mutagenesis—**To introduce mutations into the three putative nuclear localization sequences of BRCA1, a polymerase chain reaction-based strategy was used. Briefly, the following external and internal primers with HindIII restriction sites (underlined below) were used to create in-frame deletions of each NLS sequence, replacing the deletion with a single leucine residue. The external primers used for all of the NLS mutations were 5'-GATTTGGAACACTGAGAACGTTGCA (733–759 of BRCA1 cDNA) and 5'-CTTTAAGGACCCAGCTGGCGCA-GAGAA (2679–2653). For the KLP mutation, the following internal primers were used: 1A, 5'-CCTTTAAGGACCCAGCTGGC-C (2679–2653). For the KLN mutation, the following internal primers were used: 1A, 5'-CCTTTAAGGACCCAGCTGGC-C (2679–2653) and 1B, 5'-CTTTAAGGACCCAGCTGGC-C (2679–2653). The KLP and the KLN mutations were constructed by fusing BRCA11–1142 cDNAs from pBSK-BRCA1a, KLP, and KLN to the DNA-binding domain of GAL4 in the upstream activating sequence of GAL1 (UASG) (30). The BRCA1 deletion constructs in Fig. 2 were obtained by translationally fusing the DNA-binding domain of GAL4 (31, 32) in pAS (30) to cDNA fragments obtained from pBSK-BRCA1a (7) using convenient restriction sites. β-Galactosidase activity was determined by colony color and quantitated using chlorophenyl red-β-d-galactopyranoside in assays as described previously (30). Yeast Two-hybrid Screen—A cDNA library prepared from human B lymphocytes was screened as described previously (30). The protein from pAS-BRCA3.5 (see Fig. 2) served as the “bait,” which consisted of amino acids 1–1142 of BRCA1 fused to the GAL4 DNA-binding domain (31, 32) in plasmid pAS (30).

**Results**

**Determination of a Nuclear Localization Sequence in BRCA1—**To initiate the study of BRCA1 nuclear transport, we began with the identification of its NLS motif(s). By analyzing the amino acid sequences, three possible nuclear localization sequences with high charged, basic residues were found in BRCA1, 503KKKRRR5050, 606KKNNNLKRK615, and 634KKKKYN656 (Fig. 1A). To determine if these sequences are functional in nuclear localization, polymerase chain reaction-based mutagenesis (see “Experimental Procedures”) was performed that generated in-frame deletions and replacement with a single leucine residue at each of the sites (Fig. 1A). The wild-type and mutated BRCA1 proteins were tagged by fusion with the flag epitope in a pCep-based plasmid. The expression of these tagged proteins in human DU145 cells was done by transient transfection, and the proteins were detected by immunoblotting with either anti-BRCA1 mAb 6B4 (Fig. 1B, lanes 1–5) or anti-flag M2 mAb (Fig. 1B, lanes 6–10). As shown in Fig. 1B, anti-flag M2 mAb detected only ectopically expressed flag-tagged BRCA1, which co-migrates with endogenous BRCA1 as a 220-kDa protein (lanes 1–5) in each population of transfected cells (lanes 7–10) but not in untransfected cells (lane 6). This result indicated that all of the plasmid constructs transfected into DU145 cells were capable of expressing flag-tagged BRCA1 proteins, which were either wild type or mutated.

The subcellular localization of wild-type and each of the mutated proteins was determined by immunostaining with the anti-flag M2 mAb. Consistent with its previous localization (22), wild-type flag-tagged BRCA1 protein is located in the nucleus (Fig. 1C, a and b). The 634KKKYN656 mutant (Fig. 1C, g and h) is also nuclear, indicating that the residues 634KKKYN656 are not important for nuclear transport of the flag-BRCA1-KLN protein. In contrast, the KLP and KLS15 mutations both resulted in cytoplasmic localization of flag-BRCA1-KLP and flag-BRCA1-KLS proteins (Fig. 1C, e and d, and e and f, respectively), indicating that both of these stretches of basic residues are critical for nuclear import. It was noted that flag-BRCA1-KLS when overexpressed, can, in some instances, localize in the nucleus. This is illustrated in Fig. 1C, e and d, where two highly expressing cells are adjacent to each other, and one shows cytoplasmic staining and the other nuclear (arrowheads). However, flag-tagged BRCA1-KLP was never observed in the nucleus.
Identification of BRCA1-interacting Proteins—Nuclear transport of BRCA1 clearly requires interactions with other cellular proteins. We elected to use the yeast two-hybrid method to identify and clone genes encoding BRCA1-interacting proteins. Since BRCA1 has been proposed to be a transcription factor (1), it may therefore have transactivation activity. The presence of such activity would confound a two-hybrid assay. To functionally identify potential transactivation domains in BRCA1, various domains of BRCA1 protein were fused in-frame with the DNA-binding domain of GAL4 (Fig. 2) in plasmid pAS (30). If these fusion proteins contain an activation domain, they will activate the GAL4 UAS_G-responsive β-galactosidase reporter (30) after transfection into the Y153 strain. Through this analysis we defined a strong activation domain located between amino acids 1142 and 1646 (Fig. 2). This activation domain was deleted in BRCA3.5 (Fig. 2), which only contains amino acids 1–1142 of BRCA1. BRCA3.5 was then fused to the GAL4 DNA-binding domain of pAS vector as the bait for screening BRCA1-interacting proteins as described previously (30). Four different clones were isolated and sequenced. When compared with GenBank™, we found that one is novel, one has homology to an uncharacterized zinc finger domain, and two have homology to known zinc finger proteins.
domain-containing protein, and two bear sequence homology to previously cloned cDNAs (Table I). Interestingly, the sequence of hBRAP21 is identical to that of the nuclear localization signal receptor hSRP1α (26), also known as importin-α (18) or karyopherin-α (27).

Table I. Summary of clones encoding BRCA1-interacting proteins

| Clone     | Insert size | β-Galactosidase activity | Similarity with known sequences |
|-----------|-------------|--------------------------|--------------------------------|
| hBRAP2    | 0.8         | ++++                     | With sequence conserved in S. cerevisiae and Caenorhabditis elegans |
| hBRAP12   | 1.2         | +++                     | Part of an uncharacterized zinc finger domain-containing protein |
| hBRAP14   | 1.1         | ++                      | Novel sequence |
| hBRAP21   | 0.9         | ++                      | Identical to the nuclear localization signal receptor (hSRP1α/importin-α) |

* Insert size is given in kilobase pairs.

Fig. 3. Importin-α interactions with wild-type and mutant BRCA1 with nuclear localization sequence deletions. A, diagrams of the constructs used in the yeast two-hybrid assays. pAS-BRCA3.5 was described previously (Fig. 2). pAS-KLP, KLS, and KLN are shown with the coordinates for each of the mutations in BRCA1 that was fused to the DNA-binding domain (DBD) of GAL4 in pAS (30). Also illustrated is pACT-importin containing the amino acids 229–529 region of the amino acids 229–529 region of importin-α (stippled rectangle) fused to the transactivation domain of GAL4, TAD (31, 32) (solid hatched box). B, results of the assays including colony colors and levels of β-galactosidase activity. Y153 denotes the negative control cells that were not transfected.

Table II. Summary of flag-BRCA1 staining results

| Cell line | Anti-BRCA1 | M2 |
|-----------|------------|----|
| CV1       | N          | N  |
| DU145     | N          | N  |
| T24       | N          | N  |
| HBL100    | N          | N  |
| T47D      | C          | C  |
| MB468     | C          | C  |
| MDA334    | N, C       | C  |
| MB231     | C          | C  |
| ZR75      | C          | C  |
| MCF7      | N, C       | C  |

* Chen et al. (22); included for comparison with M2 staining.

† N, nucleus.

‡ C, cytoplasm.

Fig. 4. BRCA1 is located in the cytoplasm of breast cancer cells. Immunostaining (b, d, and f) of the flag-tagged BRCA1 protein in normal monkey kidney cells (CV1, a and b) and breast cancer cell lines ZR75 (e and d) and MB231 (e and f) is illustrated. DAPI staining is shown in a, c, and e to indicate nuclei of the cells.

These results are consistent with the observation that flag-BRCA1KLN is localized in nuclei (Fig. 1C, g and h). However, the KLP mutant failed to interact with importin-α and resulted in white colonies and no increase of β-galactosidase activity over background (Fig. 3B). Interestingly, a 10-fold increase in β-galactosidase activity over background was observed with the KLS mutant (Fig. 3B). As noted earlier, this increase in activity is consistent with the immunostaining data for the BRCA1 protein containing this mutation, which shows occasional nuclear localization when overexpressed (Fig. 1C, panels e and f).

Cytoplasmic Localization of Ectopically Expressed BRCA1 in Breast Cancer Cells—Previously, we transfected an expression plasmid containing flag-tagged BRCA1 into two breast cancer cell lines, T47D and MB468, and one immortalized non-breast epithelial cell line, HBL100. The flag-tagged BRCA1 protein was found in the cytoplasm of the T47D and MB468 cells and the nucleus of HBL100 cells by immunostaining with anti-flag M2 monoclonal antibody. To confirm this observation and to verify the expression of full-length flag-tagged BRCA1 protein, we repeated this experiment using four non-breast cancer and six breast cancer cell lines listed in Table II. As shown in Fig. 4 (summarized in Table II), nuclear localization of flag-BRCA1 was observed in normal monkey kidney cells CV1 (Fig. 4, a and b) and in DU145, T24, and HBL100 cells (Table II). In contrast, cytoplasmic localization of this protein is seen in ZR75 and...
MB231 (Fig. 4, c and d, and e and f, respectively) and in MB468, MDAMB361, and MCF7 breast tumor cells (Table II). These data suggest an altered transport or retention system for the BRCA1 protein in breast cancer cells.

**DISCUSSION**

BRCA1 is a nuclear protein. The identification of two regions of charged, basic amino acids between 503 and 508 and between 606 and 615 that are both crucial for efficient nuclear transport of the BRCA1 protein further supports this notion. The distance between these two motifs is much greater than the 10 amino acids separating the bipartite sites of nucleoplasmin (30). The structure and function of the NLS in BRCA1 is similar to other nuclear proteins in which two NLSs are more widely spaced such as those in the polyoma large T antigen (34), influenza A virus NS1 protein (35), and adenovirus DNA-binding protein (36). While we cannot rule out the possibility that other sequences are also required for translocation of BRCA1 from the cytoplasm to the nucleus, the NLS at 503–508 is essential for this process. This observation was supported by results showing that mutation of the NLS at 503–508 in BRCA1 completely abolished its interactions with importin-α. The NLS at 606–615 in BRCA1 is less critical, because mutation of this NLS did not completely diminish the nuclear import of BRCA1. Our results indicating that BRCA1 is a nuclear protein with a functional NLS are at odds with the report indicating that the protein is membrane-bound, and secreted (25). Such a discrepancy is puzzling but may be explained by cross-reactivity of the peptide antiserum to the epidermal growth factor receptor (37).

Using mouse polyclonal antibodies specific for the BRCA1 protein, we have consistently found BRCA1 to be a 220-kDa protein, we have circumvented the difficulties in obtaining the 220-kDa BRCA1 protein and using the specific anti-flag M2 monoclonal antibody, we have reported that the 220-kDa BRCA1 protein remains in the nucleus, perhaps BRCA1 protein is immediately exported from the nucleus, resulting in the appearance of cytoplasmic localization. Co-localization experiments similar to those in Görlich et al. (21) using normal and breast cancer cells might address this possibility.

An alternative possibility is that, in breast cancer cells, there is a problem in the regulation of the nuclear transport of BRCA1. The known mechanisms for regulating nuclear transport (reviewed in Refs. 12 and 13) are as follows: (a) phosphorylation/dephosphorylation, e.g. c-rel and v-jun and cell cycle-regulated proteins such as cyclin B-Cdk complex and pendulin; (b) cytoplasmic retention by masking of the NLSs as seen in dorsal, NF-xB, the glucocorticoid receptor, and the periodicity protein; or (c) more general regulation at the level of the nuclear pore complex. Perturbations in the gene products in any of these regulatory systems could potentially result in cytoplasmic localization of BRCA1 in breast epithelial cells. The possibility that some of the other BRCA1-interacting proteins identified in the two-hybrid screen could have this kind of role in breast cancer cells is being investigated.

Whatever the pathogenic alteration in breast cancer cells is, it is not, at this time, obvious whether the mislocation of BRCA1 is the cause of or the result of the tumor phenotype. Interestingly, there are other reports of mislocation to the cytoplasm of a nuclear tumor suppressor protein in breast and other types of cancer cells. Of 27 breast cancer cases examined, 37% demonstrated cytoplasmic staining for p53, which by sequencing was revealed to be wild type (11). In another study, wild-type p53 was found located in the cytoplasm of human cervical carcinoma cell lines with integrated human papillomavirus-18 or -16 (38). Both of these studies suggest that the tumor suppressor function of normal p53 can, in some cases, be inactivated by cytoplasmic mislocation (11, 38). These data are similar to our observations for BRCA1 and seem to suggest a global alteration of subcellular compartmentation in breast cancer cells. If this is the case, then BRCA1 and p53 along with, perhaps, other nuclear regulatory proteins may be retained in the cytoplasm of these cells, the composite effect of which may contribute to their tumorigenesis.

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