The LIM Domain Protein LMO4 Interacts with the Cofactor CtIP and the Tumor Suppressor BRCA1 and Inhibits BRCA1 Activity

Eleanor Y. M. Sum†, Benjamin Peng§, Xin Yu§, Junjie Chen§, Jennifer Byrne§, Geoffrey J. Lindeman‡‡, and Jane E. Visvader†‡ ‡‡

From †The Walter and Eliza Hall Institute of Medical Research and Bone Marrow Research Laboratories, Melbourne, Victoria 3050, Australia, §The Rockefeller University, New York, New York 10021, the ‡Mayo Clinic, Rochester, Minnesota 55905, ¶The New Children’s Hospital at Westmead, New South Wales 2145, Australia, and ‡‡Department of Medicine, University of Melbourne, Victoria 3010, Australia

LMO4 belongs to the LIM-only (LMO) group of transcriptional regulators that appear to function as molecular adaptors for protein-protein interactions. Expression of the LMO4 gene is developmentally regulated in the mammary gland and is up-regulated in primary breast cancers. Using LMO4 in a yeast two-hybrid screen, we have identified the cofactor CtIP as an LMO4-binding protein. Interaction with CtIP appeared to be specific for the LMO subclass of LIM domain proteins and could be mediated by a single LIM motif of LMO4. We further identified the breast tumor suppressor BRCA1 as an LMO4-associated protein. The C-terminal BRCT domains of BRCA1, previously shown to bind CtIP, also mediated interaction with LMO4. Tumor-associated mutations within the BRCT repeats that abolish interaction between BRCA1 and CtIP had no effect on the association of BRCA1 with LMO4. A stable complex comprising LMO4, BRCA1, and CtIP was demonstrated in vivo. The LIM domain binding-protein Ldb1 also participated in this multiprotein complex. In functional assays, LMO4 was shown to repress BRCA1-mediated transcriptional activation in both yeast and mammalian cells. These findings reveal a novel complex between BRCA1, LMO4, and CtIP and indicate a role for LMO4 as a repressor of BRCA1 activity in breast tissue.

The LIM domain is characterized by a double zinc finger structure found in proteins that have critical functions in cell fate determination, growth control, and cytoskeleton organization (reviewed in Refs. 1–4). This motif was originally identified in LIM homeodomain transcription factors and subsequently found in a variety of nuclear and cytoplasmic proteins including LIM-only (LMO),1 LIM kinase, and focal adhesion proteins. In these proteins, there are usually two or more LIM domains, which may occur in association with functionally divergent domains or by themselves, where they constitute the majority of the protein (1–4). The LIM domain functions primarily as a module for the assembly of protein complexes. There is no evidence to suggest that the LIM domain binds DNA, despite possessing similarity to the GATA-1 zinc finger motifs.

The LMO subclass of LIM proteins comprises four members (LMO1–4), each of which is defined by two tandem LIM domains (1, 5). These regulatory molecules appear to have essential functions in cell proliferation and lineage determination. LMO1 and LMO2, both translocated in acute T cell leukemia (T-ALL), are oncogenic within T cells (5). LMO2 has been demonstrated to have a central role in hematopoiesis where it is required for the development of all cell lineages (6). Furthermore, LMO2 has been established to form a multiprotein complex with the hematopoietic transcription factors SCL/TAL-1 and GATA-1 (7–9). These findings indicate a close functional relationship between LMO proteins and DNA-binding factors in blood cells.

LMO4, the most recently described member, was isolated by virtue of its interaction with the ubiquitous adaptor protein Ldb1/NL1/CLIM2 (10–13) and in an expression screen with autologous serum from a breast cancer patient (14–17). LMO4 is the most divergent member of the LMO subfamily, sharing only 50% homology with the LIM domains of other LMO proteins. The LMO4 gene is widely expressed in embryonic and adult tissues, but high levels are restricted to specific cell types (14, 15, 17).

We have recently established that the LMO4 gene is highly expressed in the proliferating mammary gland during pregnancy and that it is overexpressed in greater than 50% of primary breast cancers (18). Moreover, high levels of LMO4 were found to inhibit mammary differentiation (18). To gain insight into the mechanism by which LMO4 functions in breast epithelium, we searched for partners of LMO4 in these cells. Two interacting proteins were identified, the cofactor CtIP (CtBP-interacting protein) and the breast and ovarian tumor suppressor protein BRCA1, which has previously been shown to associate with CtIP (19–21). A multiprotein complex involving LMO4, CtIP, and BRCA1 could be demonstrated in vivo. LMO4 was found to be a repressor of BRCA1-mediated transcriptional activity, invoking a potential role for LMO4 as a negative regulator of BRCA1 function in sporadic breast cancers.

EXPERIMENTAL PROCEDURES

Plasmids—The pGBT9-LMO4 bait plasmid was generated by PCR amplification of mouse LMO4 in pSP72 using the following primers: forward, 5′-CGCGGATCCCTGGTTCATCAGGAGGCTG3′-3 and reverse, 5′-GGCGGATCCCTGTCCTCGGCAACCTG3′; the resultant product was inserted into the BamHI site of pGBT9 (CLONTECH). The first LIM domain (residues 1–82) of LMO4 was

* This work was supported by the Victorian Breast Cancer Research Consortium, by The National Breast Cancer Foundation (Australia), and by the Rotary Bone Marrow Research Laboratories, Royal Melbourne Hospital, Melbourne, Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†† To whom correspondence should be addressed. Fax: 61-3-9347-0852; E-mail: visvader@wehi.edu.au.

The abbreviations used are: LMO, LIM-only; HA, hemagglutinin; AD, activation domain; DBD, DNA-binding domain; GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; BRCT, BRCA1 C-terminal.
LMO4 Complexes with BRCA1 and CtIP

PCR-amplified using the forward primer (5′-CGCGGATCCGTAGTCTGCTACTGCTG-3′) and the reverse primer (5′-CGCGGATCCCTACCTTGGGAAAAAGCTCTGTCGTC-3′) and then cloned into the BamHI site of the FLAG-pEF1α-puro vector (22). The second LIM domain (residues 1528–1863) of LMO4 was amplified using the forward primer (5′-CGCGGATCCGTAGTCTGCTACTGCTG-3′) and the reverse primer (5′-CGCGGATCCCTACCTTGGGAAAAAGCTCTGTCGTCGTC-3′) and subsequently cloned into the BamHI site of the FLAG-pEF1α-puro vector. Expression vectors encoding Lhx1 or Lhx3 (pSV-sport-FLAG) and LMK1 were kindly provided by A. Agulnick and O. Bernard, respectively. Full-length cDNAs corresponding to the coding sequence for human LMO4 were cloned into the pEF1α-puro vector expression plasmid and a 0.7-kb mouse LMO4 cDNA fragment was cloned into pEF1α-puro vector. The expression plasmids encoding residues 45–897 of human CtIP (pCMV-HA-nS11), human BRCA1 (pcDNA3-HA-BRCA1), and mouse LMO2 (pEF1α-FLAG-LMO2) have been described previously (13, 19, 23). HA-tagged CtIP deletion mutants were generated either by PCR amplification or subcloning from pCMV-HA-nS11, into the expression vectors HA-pcDNA3.1 or HA-EF1α-puro. The SZ fragment of BRCA1 (residues 1528–1863) was recloned from pCMV-Gal4-BR-SZ (19) into HA-pcDNA3.1. Myc-tagged derivatives of wild-type and mutant BRCA1 were cloned into pcDNA3.0.3

The yeast expression construct encoding the activation domain (AD) of BRCA1. Full-length mouse LMO4 cDNA (DBD) was generated by PCR amplification of the region spanning amino acids 1293–1863 of BRCA1 and subsequent cloning into pGBT9 (CLONTECH). The mammalian Gal4DBD-AD fusion construct was generated by cloning a cDNA fragment encoding the BRCA1-AD region into pCMV-Gal4-BR (19). Full-length cDNA encoding mouse LMO4 was inserted into the yeast expression plasmid pYX212 (Ingenious). The reporter plasmid, pG5CAT, was from CLONTECH. Details of plasmid constructions are available on request.

**Yeast Two-hybrid Screen**—The pGBT9-LMO4 bait plasmid (residues 15–165) was used to screen 8.4 × 106 transformants from a primary breast adenocarcinoma cDNA library (24), following standard protocols (CLONTECH Matchmaker Two-Hybrid System).

**Antibody Production**—Full-length mouse LMO4 cDNA was amplified by PCR and subcloned into pGEX-2T (Amersham Biosciences, Inc.). The GST-LMO4 fusion protein was expressed in the bacterial strain UT5600, purified according to standard protocols (25), and used to inject rabbits to produce polyclonal antisera. The generation of rat anti-LMO4 monoclonal antibodies will be described elsewhere.

**In Vitro Binding Assay**—In vitro binding synthesis of 32P-labeled BRCA1 (C-terminal residues 1528–1863) was performed by in vitro transcription/translation of HA-pcDNA3.1-BRCA1-SZ using the TNT T7-coupled reticulocyte lysate system (Promega). Binding assays were carried out with a 10-μl aliquot of 32P-labeled BRCA1-SZ primed lysate and 100 μl (50% slurry of GST-Sepharose beads; Amersham Pharmacia) of GST-LMO4 or GST-only protein in GST interaction buffer (150 mM NaCl, 10 mM Tris, pH 8, 0.3% Nonidet P-40, 1 mM diethiothreitol, 0.25% bovine serum albumin, and 0.5 mM phenylmethylsulfonyl fluoride) for 1 h. The beads were then centrifuged briefly, washed twice in GST interaction buffer containing bovine serum albumin, followed by two more washes in GST interaction buffer without bovine serum albumin. Finally, the bound BRCA1 C-terminal polypeptide was eluted by boiling the beads for 5 min in 30 μl of loading buffer and analyzed by SDS-PAGE.

**Northern Blot Analysis**—Poly(A) RNA was isolated from human and mouse breast epithelial cell lines cited in previous studies (28) and Northern analysis was performed (29).

**Transactivation Assays in Yeast and Mammalian Cells**—The yeast transcription assay was performed in the yeast strain BJ5462; this was cotransformed with the LacZ reporter plasmid, Yep62 (generously provided by P. Vaughan), and pGBT9-BRCA1AD, and colonies selected on media deficient in Leu and Tryp. These transformants were then additionally transformed with either the pYX212-LMO4 expression plasmid or empty vector and were selected on media lacking Ura, Tryp, and Leu. β-Galactosidase activities were determined using the o-nitrophenyl-β-D-galactoside liquid culture assay following standard protocol (CLONTECH Yeast Protocols Handbook).

Kidney embryonal 293T cells were transiently transfected (six-well plates) with the indicated plasmids: 0.5 μg of pG5CAT (CLONTECH), 1 μg of pCMV-Gal4b-BRCA1-AD, or 1 μg of Gal4b parental vector, and either 2 μg of FLAG-pEF1α-LMO4 or 2 μg of empty control vector using the calcium phosphate precipitation method. CAT activity was determined using the CAT enzyme-linked immunosorbent assay system (Roche Molecular Biochemicals) and was normalized against protein concentration, as determined by the Bradford assay (Bio-Rad).

**RESULTS**

**Identification of CtIP as a LMO4-interacting Protein**—We used the yeast two-hybrid system to identify LMO4-interacting proteins in breast epithelium. A screen of 8.4 × 106 transformants of a primary breast adenocarcinoma cDNA library yielded more than 800 His+ colonies. Six-hundred and fifty-nine β-galactosidase-positive clones were isolated and sequen-tially screened by yeast colony hybridization using cDNA probes representing the known LMO4-associated proteins, Ldb1 and deformed epidermal autoregulatory factor (DEAF1) (14–16), and known false positives. Approximately 60% of these clones corresponded to Ldb1/Ldb2 (30%) or DEAF1 (30%). Of 150 cDNA clones sequenced, 22 were found to correspond to either Ldb1 or Ldb2, 20 encoded DEAF1, at least 70 corresponded to false positives (e.g. ribosomal, mitochondrial, and extracellular matrix proteins), while the remaining 38 cDNAs represented 9 distinct genes or expressed sequence tags. One of these clones corresponded to the complete coding sequence of CtIP (CtBP-interacting protein), which encodes a cofactor originally identified on the basis of its interaction with the transcriptional corepressor CtBP (adenovirus E1A C-terminal-binding protein) (30).

**In Vivo Association between LMO4 and CtIP**—A specific interaction between CtIP and LMO4 was confirmed in mammalian cells. Expression vectors encoding LMO4, LMO2, or heterologous LIM domain proteins, each carrying a FLAG or Myc epitope at the N terminus, were coexpressed in COS cells with an expression vector harboring CtIP (residues 45–897), were transiently transfected into 293T cells. Whole cell extracts were analyzed using a coupled coimmunoprecipitation/Western blot assay. As shown in Fig. 1A, FLAG-LMO4 and CtIP were found to specifically associate in vivo, in reciprocal coimmunoprecipitation experiments (lanes 1 and 4). CtIP was not detected in immunoprecipitates from cells expressing FLAG-LMO4 alone (lane 3) or in those using an isotype-matched control antibody. A single LIM domain of LMO4 (amino acids 1–82) was found to be sufficient to mediate interaction with CtIP in mammalian cells (Fig. 1B, lane 1). Only the first LIM domain of LMO4, but

---

2 J. Chen, unpublished data.

3 E. Y. M. Sum, G. J. Lindeman, and J. E. Visvader, unpublished data.
not the second LIM domain (amino acids 79–165), could associate with Ctip (lane 2). Ctip also coprecipitated with the related LIM domain protein, LMO2 (Fig. 1A, lane 2), but not with the nuclear LIM homeodomain proteins Lhx1 and Lhx3 (Fig. 1C, lanes 2 and 3), nor with LIM kinase (LMK1) (Fig. 1C, lane 4). To delineate the domains within Ctip that mediate interaction with LMO4, a series of Ctip deletion mutants (Fig. 2A), each linked to an N-terminal HA-epitope tag, were cotransfected with FLAG-LMO4 into 293T epithelial cells. As shown in Fig. 2B, HA-Ctip (amino acids 45–371), HA-Ctip (amino acids 371–535 of Ctip), and HA-Ctip (amino acids 536–794 of Ctip) all interacted with LMO4. However, HA-Ctip (amino acids 795–1094 of Ctip) did not interact with LMO4. Western blot analysis of lysates confirmed expression of Ctip and LMO4 proteins (lower panels). D, interaction between endogenous LMO4 and Ctip proteins in HBL100 epithelial cells. Nuclear extracts were immunoprecipitated with preimmune serum (lane 1) or anti-LMO4 antisera (lane 2), then blotted using anti-Ctip monoclonal antibody. Nuclear extract from these cells was loaded in an adjacent lane to provide a size control for Ctip (lane 3).

LMO4 Complexes with BRCA1 and CtIP

not the second LIM domain (amino acids 79–165), could associate with Ctip (lane 2). Ctip also coprecipitated with the related LIM domain protein, LMO2 (5) (Fig. 1A, lane 2), but not with the nuclear LIM homeodomain proteins Lhx1 and Lhx3 (Fig. 1C, lanes 2 and 3), nor with LIM kinase (LMK1) (Fig. 1C, lane 4).

To delineate the domains within Ctip that mediate interaction with LMO4, a series of Ctip deletion mutants (Fig. 2A), each linked to an N-terminal HA-epitope tag, were cotransfected with FLAG-LMO4 into 293T epithelial cells. As shown in Fig. 2B, HA-Ctip (amino acids 45–371), HA-Ctip (amino acids 371–535 of Ctip), and HA-Ctip (amino acids 536–794 of Ctip) all interacted with LMO4. However, HA-Ctip (amino acids 795–1094 of Ctip) did not interact with LMO4. Western blot analysis of lysates confirmed expression of Ctip and LMO4 proteins (lower panels). D, interaction between endogenous LMO4 and Ctip proteins in HBL100 epithelial cells. Nuclear extracts were immunoprecipitated with preimmune serum (lane 1) or anti-LMO4 antisera (lane 2), then blotted using anti-Ctip monoclonal antibody. Nuclear extract from these cells was loaded in an adjacent lane to provide a size control for Ctip (lane 3).

LMO4 Interacts with Two Distinct Domains in Ctip—The function of Ctip is not known but it appears to serve as a cofactor for several nuclear regulatory proteins, including BRCA1, adenovirus E1A C-terminal-binding protein (CtBP), retinoblastoma, and p130 pocket proteins (9–19, 30–32). In addition to the defined regions that interact with these proteins, Ctip contains two potential leucine zipper domains (amino acids 120–141 and 740–761) (31), depicted in Fig. 2A. To delineate the domains within Ctip that mediate interaction with LMO4, a series of Ctip deletion mutants (Fig. 2A), each linked to an N-terminal HA-epitope tag, were cotransfected with FLAG-LMO4 into 293T epithelial cells. As shown in Fig. 2B, HA-Ctip (amino acids 45–371), HA-Ctip (amino acids 371–535 of Ctip), and HA-Ctip (amino acids 536–794 of Ctip) all interacted with LMO4. However, HA-Ctip (amino acids 795–1094 of Ctip) did not interact with LMO4. Western blot analysis of lysates confirmed expression of Ctip and LMO4 proteins (lower panels). D, interaction between endogenous LMO4 and Ctip proteins in HBL100 epithelial cells. Nuclear extracts were immunoprecipitated with preimmune serum (lane 1) or anti-LMO4 antisera (lane 2), then blotted using anti-Ctip monoclonal antibody. Nuclear extract from these cells was loaded in an adjacent lane to provide a size control for Ctip (lane 3).
formed using antibody. After SDS-PAGE electrophoresis, Western blotting was performed with expression constructs encoding HA-tagged derivatives of CtIP (lanes 1–5), together with that encoding FLAG-tagged LMO4. Lysates were prepared and proteins immunoprecipitated using anti-FLAG monoclonal antibody (F) or control isotype-matched (C) monoclonal antibody. After SDS-PAGE electrophoresis, Western blotting was performed using α-HA antibody, specific for CtIP protein. Western blot analysis confirmed expression of FLAG-LMO4 and CtIP mutant proteins (lower panels).

897), HA-CtIP (amino acids 45–897), and HA-CtIP (amino acids 620–897) proteins could associate with FLAG-LMO4 (lanes 2, 4, 5, and 6). In contrast, neither the HA-CtIP (amino acids 59–320) nor HA-CtIP (amino acids 281–620) mutants were immunoprecipitable by an anti-FLAG antibody (lanes 1 and 3, respectively). Thus, there are apparently two regions within CtIP that can mediate interaction with LMO4: a small domain at the N terminus (residues 45–59) and a C-terminal region (residues 620–897), which encompasses a putative leucine zipper motif (Fig. 2A). These regions are distinct from those that associate with CtBP (amino acids 392–396), retinoblastoma (amino acids 153–157), and BRCA1 (amino acids 133–369) (19, 27, 30–32).

**Coexpression of LMO4 and CtIP in Breast Epithelial Cell Lines**—Both the LMO4 and CtIP genes have been reported to be expressed in a number of different tissues and cell types (14, 15, 20). We surveyed their expression in a panel of breast epithelial cell lines, the majority of which were derived from human breast cancers but also included immortalized human cells (184). Northern analysis revealed CtIP RNA (3.6 kb) levels were relatively uniform, while expression of the LMO4 transcripts (1.8 and 2.3 kb) varied dramatically (Fig. 3). High levels of LMO4 were apparent in a number of human breast cancer cell lines, including BT-549, BT-474, HS-578T, MDA-MB361, T-47D, and ZR-75B (Fig. 3), relative to the low levels evident in the immortalized 184 cells, as we reported recently (18). In human breast cancers, overexpression of the LMO4 gene has been observed at both the RNA and protein levels (18).

**LMO4 Also Interacts with the Breast and Ovarian Tumor Suppressor BRCA1**—Since CtIP was recently demonstrated to interact with the breast tumor suppressor BRCA1 (19–21), we investigated whether LMO4, CtIP, and BRCA1 could participate in a multiprotein complex. Both CtIP and LMO4 were coimmunoprecipitated with an anti-Myc antibody from 293T cells expressing Myc-tagged BRCA1, FLAG-tagged LMO4 and CtIP (Fig. 4A, lane 2). This finding revealed that all three proteins can form a stable multiprotein complex in vivo. The presence of exogenous CtIP was not necessary for immunoprecipitation of LMO4 by the anti-Myc antibody, as shown in Fig. 4A (lane 1). These results raised the possibility that LMO4 might directly associate with BRCA1 (see below).

To further examine this interaction in epithelial cells, a rat anti-LMO4 monoclonal antibody was used to immunoprecipitate proteins from HBL100-derived nuclear extracts. This antibody specifically recognizes a 17-kDa protein in cells transfected with a LMO4 expression vector but not in those lacking LMO4. Endogenous BRCA1 was immunoprecipitated by the anti-LMO4 monoclonal antibody (Fig. 4B, lane 2), but not with a control antibody (Fig. 4B, lane 3). This result confirms an in vivo association between LMO4 and BRCA1 and, moreover, demonstrates that this interaction occurs between native proteins in epithelial cells. Immunoblotting of the anti-LMO4 immunoprecipitated protein with anti-CtIP monoclonal antibody yielded a faint band of 125 kDa, corresponding to CtIP (Fig. 4B, middle panel), while blotting with anti-LMO4 antibody gave rise to the expected 17-kDa LMO4 protein (Fig. 4B, lower panel). Thus, LMO4, BRCA1, and CtIP have the potential to form a native complex in vivo.

We investigated whether the nuclear adaptor protein Ldb1, which binds LMO4 and other LIM proteins with high affinity (14–16), could also participate in the multiprotein complex. Ldb1 could be immunoprecipitated from cells transfected with plasmids encoding CtIP, BRCA1, LMO4, and Ldb1 using an anti-CtIP antibody (Fig. 4C). Therefore, all four proteins have the potential to form a stable complex in vivo.

The **BRCT Domain of BRCA1 Directly Interacts with LMO4**—The C-terminal 335 amino acids of BRCA1 (SZ fragment, residues 1528–1863) were sufficient to mediate interaction with LMO4 (Fig. 5A) in transfected cells. This region encompasses two tandem BRCT motifs that are required for
anti-CtIP antibody. As a control, immunoprecipitate was also blotted with anti-LMO4 monoclonal antibody (lane 2) or absence of plasmid encoding CtIP (lane 1). Lysates were prepared and proteins were immunoprecipitated with anti-Myt or control (C) monoclonal antibody, then fractionated by electrophoresis, before blotting with either anti-FLAG or anti-CtIP antibody. Expression of individual proteins in these cell extracts was confirmed by Western blotting (data not shown). B, endogenous LMO4, BRCA1, and CtIP proteins associate in HBL100 epithelial cells. Nuclear lysates were immunoprecipitated with a rat anti-LMO4 monoclonal (upper panel) or anti-CtIP (middle panel) monoclonal antibody. The immunoprecipitate was divided between two SDS-polyacrylamide gels, one of which underwent extended electrophoresis for detection of BRCA1 (220 kDa), while the other was immunoblotted with anti-CtIP antibody. As a control, immunoprecipitate was also blotted with anti-LMO4 monoclonal antibody (lower panel). Lysate from HBL100 cells was loaded in an adjacent lane to provide a size control for the respective proteins (lane 1), indicated by arrows. C, interaction between LMO4, CtIP, BRCA1, and Ldb1 in mammalian cells. Extracts derived from 293T cells transfected with expression vectors encoding BRCA1, Flag-tagged LMO4, CtIP, and Ldb1 were immunoprecipitated with anti-CtIP antibody (lane 2) or control antibody (lane 3), then immunoblotted with the indicated antibodies. Western blot analysis confirmed high level expression of individual proteins in these transfectants (lane 1).

**FIG. 4.** LMO4 forms a complex with CtIP and BRCA1 in vivo and directly interacts with BRCA1. **A**, specific interaction between LMO4, CtIP, and BRCA1 in vivo. 293T cells were transfected with expression constructs encoding FLAG-tagged LMO4 and Myc-tagged BRCA1 in the presence (lane 2) or absence of plasmid encoding CtIP (lane 1). Lysates were prepared and proteins were immunoprecipitated with anti-FLAG (F) or control (C) antibody, then Western blotted with anti-Myc or control antibody. The immunoprecipitate was divided between two SDS-polyacrylamide gels, one of which underwent extended electrophoresis for detection of BRCA1 (220 kDa), while the other was immunoblotted with anti-CtIP antibody. As a control, immunoprecipitate was also blotted with anti-LMO4 monoclonal antibody (lower panel). Lysate from HBL100 cells was loaded in an adjacent lane to provide a size control for the respective proteins (lane 1), indicated by arrows. **B**, interaction between LMO4, CtIP, BRCA1, and Ldb1 in mammalian cells. Extracts derived from 293T cells transfected with expression vectors encoding BRCA1, Flag-tagged LMO4, CtIP, and Ldb1 were immunoprecipitated with anti-CtIP antibody (lane 2) or control antibody (lane 3), then immunoblotted with the indicated antibodies. Western blot analysis confirmed high level expression of individual proteins in these transfectants (lane 1).

**FIG. 5.** The C-terminal BRCT domains of BRCA1 mediate interaction with LMO4. **A**, 293T cells were transfected with expression constructs encoding FLAG-tagged LMO4 and the C-terminal region (SZ) of BRCA1 carrying an HA-tag. Lysates were immunoprecipitated with anti-FLAG (F) or control (C) antibody, then Western blotted with anti-HA antibody. **B**, LMO4 and the BRCT domain interact in vitro. In vitro translated [35S]methionine-labeled BRCA1-SZ polypeptide (amino acids 1528–1863) was incubated with GST-LMO4 fusion protein, immobilized on glutathione-Sepharose beads, was incubated with in vitro translated [35S]methionine-labeled BRCA1-SZ polypeptide (amino acids 1528–1863). As shown in Fig. 5B, BRCA1 specifically associated with GST-LMO4, but failed to interact with GST alone. **C**, BRCA1 specifically associated with GST-LMO4 but failed to interact with GST alone. To exclude the possibility that CtIP was acting as a bridging molecule between LMO4 and BRCA1, we tested the interaction between BRCA1 and LMO4 in yeast. It is notable that CtIP appears to be absent from the Saccharomyces cerevisiae genome (Saccharomyces Genome Database, NCBI). Cotransformation of H7c cells with the SZ portion of BRCA1 linked to the Gal4 activation domain and GBT9-LMO4 revealed a direct association between these proteins (Fig. 5C). His+ β-Gal+ colonies were obtained for these transformants, as was observed for yeast cells cotransformed with LMO4 and CtIP. Thus, the BRCT domains of BRCA1 mediate direct binding to both LMO4 and CtIP.

**Tumor-derived Mutations in BRCA1 Do Not Affect LMO4 Binding**—Tumor-associated mutations have been identified in a significant proportion of hereditary breast cancer patients. The lesions include missense mutations (P1749R, M1775R) and a nonsense mutation (Y1853A) that deletes the C-terminal 11 amino acids of BRCA1 (19, 21). These tumor-associated mutations have been demonstrated to abolish binding of BRCA1 to CIP (19, 21). To assess their effect on BRCA1-LMO4 interaction, Myc-tagged BRCA1 expression constructs containing these mutations were tested for their ability to bind FLAG-tagged LMO4 in 293T cells. All mutants were found to interact with LMO4, as observed for the wild-type BRCA1 polypeptide (Fig. 6). Thus, mutations occurring within BRCA1 in hereditary breast cancer patients can abolish CIP binding without affecting interaction between BRCA1 and LMO4.

**LMO4 Represses the Transcriptional Activity of BRCA1**—The BRCT domain has previously been shown to activate tran-
scription when recruited to a promoter via a heterologous DNA-binding domain (33, 34). Further characterization of the C terminus has revealed that there are two adjacent activation domains, AD1 (residues 1293–1558) and AD2 (residues 1560–1863), which together constitute the AD domain of 571 amino acids (35). Functional synergy occurs between AD1 and AD2 in both yeast and mammalian cells, such that the AD region is a stronger transcriptional activator than either domain alone (35). To explore the effect of LMO4 on activation of transcription by BRCA1, we initially used a yeast-based transcription assay with the C-terminal AD region of BRCA1 fused to the Gal4 DNA-binding domain. This plasmid was introduced with either a LMO4 yeast expression vector or an empty vector into the BJ5462 yeast strain, harboring a LacZ reporter plasmid. BRCA1-AD was a potent transactivator, inducing transcription 400–600-fold over basal Gal4 DNA-binding activity (Fig. 7A). This activation was markedly repressed by LMO4, which resulted in an 80% reduction in the level of activation by BRCA1-AD (Fig. 7A). The expression of BRCA1-AD was equivalent in the presence or absence of the LMO4-encoding plasmid (Fig. 7A, lower panel).

To confirm the findings of the yeast transcription assay, we performed a similar study in mammalian 293T cells. BRCA1-AD tethered to the Gal4 DNA-binding domain induced transcription of the Gal4-CAT reporter gene by 3–4-fold over the basal activity of the Gal4-DNA-binding domain alone (Fig. 7B). Consistent with the findings above, LMO4 exerted a negative effect on transactivation by BRCA1 (~3-fold), returning transcriptional activity to the basal level observed with the Gal4-DBD alone. LMO4 does not act as a general repressor, since the activity of the VP16 transactivation domain was not altered by forced LMO4 expression (data not shown). Moreover, the basal activity of the Gal4-DBD was not reduced by the presence of LMO4 (Fig. 7B). The expression of BRCA1-AD was unaffected by the presence of LMO4 (Fig. 7B, lower panel).

DISCUSSION

Our recent findings implicate LMO4 as an important regulator of cell proliferation. LMO4 is most highly expressed in the developing mammary gland during pregnancy and forced expression of this gene inhibits differentiation of mammary epi-

![Fig. 6. Tumor-associated mutants of BRCA1 interact with LMO4. 293T cells were transfected with expression constructs encoding Flag-tagged LMO4 together with myc-tagged wild-type BRCA1 (lane 1) or mutants of BRCA1 (lanes 2–4). Lysates were prepared and proteins immunoprecipitated with anti-FLAG (F) or control (C) monoclonal antibody. Immunoblotting with anti-Myc antibody revealed interactions between LMO4 and all BRCA1 derivatives.](http://www.jbc.org/)

![Fig. 7. LMO4 represses BRCA1 activity in both yeast and mammalian transcriptional activation assays. A, activation of β-galactosidase expression in BJ5462 cells expressing either GAL4-DBD (DNA-binding domain) or GAL4-BRCA1-AD (activation domain) fusion protein in the presence or absence of a yeast LMO4 expression vector. Transcriptional activity is expressed relative to that of the BRCA1-AD domain, which was designated to be 100%. B, activity of the BRCA1 activation domain in 293T cells cotransfected with the pG5CAT reporter plasmid, containing five Gal-binding sites upstream of the chloramphenicol acetyltransferase gene, and either an LMO4 expression construct or empty vector. The BRCA1-AD domain was fused to the GAL4-DBD. Basal activity of the GAL4-DBD is shown in both yeast and mammalian cells. The data in A and B represent an average of three independent experiments with standard error of the mean indicated. Western blot analysis confirmed expression of the relevant proteins, designated by arrows, in yeast or mammalian cells.](http://www.jbc.org/)
LMO4 Complexes with BRCA1 and CtIP

thelial cells. Significantly, overexpression of LMO4 was demonstrated in a high proportion of primary breast cancers, suggesting a role for LMO4 in the pathogenesis of cancer (18). However, the mechanism by which LMO4 functions is not yet known. Here we report the identification of a novel complex involving LMO4, CtIP, and BRCA1. This observation is of particular interest as BRCA1 is a tumor suppressor in familial breast cancer and CtIP has been shown to interact directly with BRCA1 (19–21, 27). A native complex comprising LMO4, CtIP, and BRCA1 was demonstrated to exist in vivo. We describe a function for the LMO4-BRCA1 protein complex, whereby LMO4 was found to repress transactivation by BRCA1 (Fig. 7).

The BRCA1 tumor suppressor gene is involved in critical cellular processes, including DNA repair, cell cycle control, and transcriptional regulation (36–39). This 220-kDa protein acts as a scaffold for multiple protein-protein interactions. There are two recognizable protein motifs in BRCA1, an N-terminal RING domain (40) and a C-terminal BRCT domain (41), that comprise two tandem BRCT motifs. Both of these regions are required for BRCA1-mediated tumor suppression. Several transcriptional regulators have been shown to interact, either directly or indirectly, with the BRCT motifs. These include p53, p300, CBP, retinoblastoma, CtIP, and histone deacetylase (reviewed in Refs. 38 and 39). LMO4 also utilizes the BRCT region to bind BRCA1. The BRCT domain harbors an acidic transactivation domain that appears to recruit RNA PolII holoenzyme to bind BRCA1. The BRCT domain harbors an acidic transactivation domain (42) to confer transactivation activity. Recently, an extended transactivation domain (AD), encompassing the BRCT domain plus a proximal region, was identified (35). LMO4 attenuates BRCA1-mediated transcriptional activation via the AD domain. The BRCA1 activity detected in the GAL4 fusion assay may reflect an important function distinct from gene-specific transcription, such as chromatin remodeling, an activity reported for the BRCT region of BRCA1 (43). The precise BRCA1 function impaired by LMO4 is yet to be established.

LMO4 and CtIP both interact with the BRCT domain of BRCA1. The precise contact residues are likely to differ since tumor-derived mutations in the BRCT domain abolish BRCA1’s interaction with CtIP (19, 20) but not with LMO4. CtIP has been demonstrated to repress transcription when recruited to a promoter by the Gal4 DNA-binding domain, suggesting that it is a corepressor (32). Moreover, it has been reported to repress BRCA1-mediated transactivation of the p21 promoter (21) and when recruited to a GAL4-dependent promoter (44). Although LMO4 interacts with two regions of CtIP, it does not further repress transcription by CtIP on the Gal4 promoter, in contrast to its effect on BRCA1 activity. Therefore, LMO4 is unlikely to be a general corepressor, consistent with the finding that it does not inhibit the basal activity of a Gal4-dependent promoter or alter activity of the VP16 transactivation domain (Fig. 7 and data not shown).

There is increasing evidence that the LIM domain acts as a docking site for the assembly of multiprotein complexes (1, 2). This motif can mediate interaction with other LIM domains or with heterologous domains. The LIM-only protein LMO2 has been shown to physically associate with Ldb1 and the transcription factors GATA-1, a zinc finger protein, and SCL, a basic helix-loop-helix factor (7–9). Evidence that this complex is physiologically significant has emerged from the findings that LMO2, SCL, and GATA-1 are all essential for erythropoiesis (reviewed in Ref. 45). LMO4 also interacts with a number of proteins. In addition to CtIP and BRCA1, it associates with the adaptor protein Ldb1 and the transcription factor DEAF1 (15). Ldb1 appears to participate in formation of the multiprotein complex between LMO4, BRCA1, and CtIP, but the functional relevance of the DEAF1-LMO4 association is not yet known.

LIM domains can exert both positive and negative effects. In LIM homeodomain proteins, the LIM domains have been implicated in the inhibition of DNA binding (46, 47) but have also been shown to be required for synergistic activation of genes by interacting with heterologous transcription factors (48, 49). In the LIM-only protein family, LMO2 acts as a negative regulator of erythroid and T cell differentiation (5, 13), while LMO4 is an inhibitor of mammary epithelial differentiation (18). These observations suggest that inappropriate expression of LMO2 or LMO4 maintains the undifferentiated state and has implications for tumorigenesis, in which they may act in an analogous manner. The molecular mechanisms underlying inhibition of differentiation or LMO4 repression of BRCA1 function remain to be defined. LMO proteins may sequester cofactors or DNA-binding transcription factors and consequently alter their DNA binding potential. Alternatively, they may directly contribute an activation or repression domain to influence transcriptional activity.

LMO proteins have been implicated in oncogenesis. LMO1 and LMO2 have been established as T cell oncoproteins (5), while LMO4 is overexpressed in more than 50% of sporadic primary breast cancers and in ~40% of noninvasive ductal carcinoma in situ (18). The identification of targets of LMO4 is a prerequisite to defining the pathways perturbed in those breast cancers overexpressing LMO4. Our data imply that BRCA1 may be one target in breast epithelial cells. BRCA1 is inactivated by either truncation or missense mutation in a significant proportion of familial breast cancer cases (50, 51). Although mutations of BRCA1 are rare in sporadic cancers, expression of this gene has been found to be reduced or undetectable in the majority of high grade carcinomas (52, 53). Epigenetic loss of BRCA1 may occur by allelic loss (54, 55) or at the transcriptional or postranscriptional level (52). There is evidence indicating that BRCA1 expression is suppressed by hypermethylation in sporadic breast carcinomas (56–59). We speculate that overexpression of LMO4 in a subset of sporadic tumors represents another mechanism by which to down-regulate BRCA1 activity and may contribute to the pathogenesis of breast cancer.

Acknowledgments—We are indebted to R. Baer and P. Vaughan for reagents and invaluable advice. We are also grateful to K. Hahm and L. Santamaria for excellent assistance; B. Mesiti for help with graphics; and A. Agulnick, D. Huang, K. Khanna, O. Bernard, H. Puthalakath, G. Chinnadurai, and S. Nicholson for constructs.

REFERENCES
1. Sanchez-Garcia, I., and Rabbits, T. H. (1994) Trends Genet. 10, 315–320
2. Dawid, I. B., Breen, J. J., and Toyama, R. (1998) Trends Genet. 14, 156–162
3. Bach, I. (2000) Mech. Dev. 91, 5–17
4. Jurata, L. W., and Gill, G. N. (1998) Curr. Top. Microbiol. Immunol. 228, 75–113
5. Rabbits, T. H. (1998) Genes Dev. 12, 2651–2657
6. Yamada, Y., Warren, A. J., Dobson, C., Forster, A., Pannell, R., and Rabbits, T. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3890–3895
7. Valge-Archer, V. E., Osada, H., Warren, A. J., Forster, A., Li, J., Baer, R., and Rabbits, T. H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8617–8621
8. Wadman, I., Li, J., Bach, R. D., Forster, A., Osada, H., Rabbits, T. H., and Baer, R. (1994) EMBO J. 13, 4831–4839
9. Wadman, I. A., Osada, H., Grutz, G. G., Agulnick, A. D., Westphal, H., Forster, A., and Rabbits, T. H. (1997) EMBO J. 16, 3145–3157
10. Agulnick, A. D., Taira, M., Breen, J. J., Tanaka, T., Dawid, I. B., and Westphal, H. (1996) Nature 384, 270–272
11. Jurata, L. W., Kenny, D. A., and Gill, G. N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11693–11698
12. Bach, I., Carriere, C., Ostendorff, H. P., Andersen, B., and Rosenfeld, M. G. (1997) Genes Dev. 11, 1370–1380
13. Visvader, J. E., Mao, X., Fujiwara, Y., Hahm, K., and Orkin, S. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13707–13712
14. Kenny, D. A., Jurata, L. W., Saga, Y., and Gill, G. N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11257–11262
15. Sugihara, T. M., Bach, I., Kioussi, C., Rosenfeld, M. G., and Andersen, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15418–15423
The LIM Domain Protein LMO4 Interacts with the Cofactor CtIP and the Tumor Suppressor BRCA1 and Inhibits BRCA1 Activity
Eleanor Y. M. Sum, Benjamin Peng, Xin Yu, Junjie Chen, Jennifer Byrne, Geoffrey J. Lindeman and Jane E. Visvader

J. Biol. Chem. 2002, 277:7849-7856.
doi: 10.1074/jbc.M110603200 originally published online December 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110603200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 58 references, 24 of which can be accessed free at
http://www.jbc.org/content/277/10/7849.full.html#ref-list-1