Cloning Genes Encoding MHC Class II–Restricted Antigens: Mutated CDC27 as a Tumor Antigen

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In an effort to identify tumor-specific antigens recognized by CD4$^+$ T cells, an approach was developed that allows the screening of an invariant chain–complementary DNA fusion library in a genetically engineered cell line expressing the essential components of the major histocompatibility complex (MHC) class II processing and presentation pathway. This led to the identification of a mutated form of human CDC27, which gave rise to an HLA-DR4–restricted melanoma antigen. A mutated form of triosephosphate isomerase, isolated by a biochemical method, was also identified as an HLA-DR1–restricted antigen. Thus, this approach may be generally applicable to the identification of antigens recognized by CD4$^+$ T cells, which could aid the development of strategies for the treatment of patients with cancer, autoimmune diseases, or infectious diseases.

CD4$^+$ T cells play a central role in antitumor immune responses and autoimmune and infectious diseases (*1–3*). Identification of the antigens (Ags) recognized by CD4$^+$ T cells can provide opportunities for the development of cancer vaccines and strategies for the treatment of patients with autoimmune and infectious diseases. CD4$^+$ T cells recognize peptides bound to MHC class II molecules on the surface of Ag-presenting cells. Assembly of the α and β chains of MHC class II molecules as well as their association with the invariant chain (Ii) begins in the endoplasmic reticulum. A targeting sequence in the cytoplasmic tail of Ii is responsible for transport of nonameric (αβIi)3 complexes from the endoplasmic reticulum to intracellular compartments with endosomal or lysosomal characteristics and ultimately to acidic endosomal- or lysosomal-like structures called MHC class II compartments (*4, 5*). Several approaches have been tried to identify Ags recognized by MHC class II–restricted CD4$^+$ T cells (*6–8*), but a generally applicable method has not emerged.

Our approach was to generate a cell line with a high transfectability as well as with the ability to process and present class II–restricted Ags to T cells, and to create an Ii fusion library for targeting Ii fusion proteins to the endosomal or lysosomal compartment for efficient Ag processing. We genetically engineered HEK 293 cells, a transformed human kidney embryonic cell line, to express DRα, DRβ, DMA, DMB, and Ii (*9*), which are required for processing and presentation of class II–restricted Ags (*4, 5, 10*). The resultant line 293IMDR1 expressed DMA, DMB, and Ii in addition to DRα and DRβ1*0101; 293IMDR4 cells expressed the same set of genes except DRβ1*0401; and 293DR4 cells expressed only DRα and DRβ1*0401.

We also made Ii-cDNA fusions such that the translated fusion proteins could be targeted to MHC class II compartments where antigenic peptides could be generated by proteolytic degradation of the Ii fusion proteins and loaded onto MHC class II molecules. We tested this hypothesis by using the hemagglutinin (HA) Ag from influenza A virus and constructing several HA–green fluorescent protein (GFP) fusion genes with or without various lengths of Ii (*11, 12*). After analyzing the ability of 293IMDR4 cells to process and present HA peptides derived from the various Ii fusion constructs and examining the intracellular localization of HA (*12, 13*), we chose the Ii(1–80) fusion construct to make Ii fusion libraries. This choice was supported by previous studies showing the critical role of Ii fusion in enhancing the presentation of Ags through the MHC class II pathway (*14*).

We evaluated the utility of this system for the identification of unknown MHC class II–restricted melanoma Ags recognized by CD4$^+$ T cells. CD4$^+$ tumor-infiltrating lymphocytes (TIL) cultured from an intramuscular metastatic lesion in patient 1359 could recognize autologous whole 1359mel tumor cells, but not MHC class II–positive HEK 293 cells, autologous Epstein-Barr virus (EBV)–B cells (1359EBV), nor other MHC class II–positive EBV-B and tumor cell lines (Fig. 1A). CD4$^+$ TIL1359 did not recognize cell lysates of 1359mel pulsed onto 1359EBV-B cells (*13*), thus excluding the possibility of identifying the Ag by a biochemical purification approach. T cell recognition of 1359mel was HLA-DR–restricted because inhibition was observed with antibody L243 to DR (Fig. 1B). DR molecules expressed by patient 1359 included β1*0301, β1*0401, β3*0101, and β4*0101. We therefore used HLA-DRβ1*0401 as the Ag-presenting molecule for the cDNA library screening.

The vector pTSX was constructed, which allowed the fusion of 1359mel cDNAs to a gene fragment encoding the first 80 amino acids of Ii (Fig. 1C) (*15*). After screening the cDNA library, we isolated six positive cDNA clones (*16, 17*). The CD4$^+$ TIL1359 recognized 293IMDR4 transfected with the positive cDNA E12-6, but not with a control cDNA, nor 293IMDR1 transfected with either cDNA E12-6 or control cDNA, suggesting that T cells recognized an Ag encoded by E12-6 in the context of HLA-DR4 (Fig. 1D).

DNA sequencing analysis indicated that all six positive clones contained an identical cDNA insert, which was a partial cDNA encoding the human CDC27 protein involved in cell cycle regulation (*18*). A point mutation (C→T) at amino acid position 711 resulted in a nonconservative substitution of leucine for serine. The C→T mutations were identified in the MHC class I–restricted mutated melanoma Ags CDK4 and β-catenin recognized by CD8$^+$ cytolytic T lymphocytes (*19, 20*), suggesting that the C→T mutation frequently occurs in malignant melanomas and may be a consequence of ultraviolet light exposure (*21*).

To identify the peptide epitope from CDC27 recognized by CD4$^+$ TIL1359, we made five 15-residue peptides spanning the segment containing the mutation (*22*), but none of them was recognized (*13*). Because CD4$^+$ TIL1359 recognized 293IMDR4 transfected with a plasmid containing Ii fused with the COOH-terminus of CDC27, 15 more 15-residue peptides overlapping by five amino acids were tested for recognition. One of these peptides starting at amino acid 758 to 772 was recognized by CD4$^+$ TIL1359 (*13*). The defined minimal peptide sequence was FSWAMDLDPKGA (*23*) required for T cell recognition (Fig. 2A), and the sequence suggested that Phe (F) at amino acid position 760 was the P1 anchor residue for MHC binding, conforming to the HLA-DR4 binding motif (*24*). Substitution of lysine (K) with phenylalanine (F) at position 769 (FSWAMDLDP*F*GA) resulted in a loss of T cell reactivity (Fig. 2A), indicating that this residue might be involved in a T cell receptor contact. Peptide titration experiments showed that TIL1359 could recognize the 12-residue peptide at 100 ng/ml (Fig. 2B).

Because CD4$^+$ TIL1359 recognized a nonmutated CDC27 epitope expressed in 1359mel but not in other MHC class II matched tumors or EBV-B cells (Fig. 1A), we examined whether T cell recognition of 1359mel was due to overexpression or protein accumulation of

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CDC27 in 1359mel. Protein immunoblot analysis (*25*) showed that expression of CDC27 in 1359mel was equal to or slightly lower than that in other tumor lines tested (Fig. 3A). Although HLA-DR4$^+$ HEK 293 cells expressed more CDC27 protein, they did not stimulate TIL1359, suggesting that there was no correlation between the amount of CDC27 protein and T cell recognition. DNA sequence analysis revealed that the C→T mutation was present in the 1359mel cell line and fresh (primary) tumor 1359FT, but not in the autologous 1359EBV-B cells, nor in 1102mel or HEK 293 cells (*13*). Thus, it appeared that the Ser^{711}→Leu mutation was responsible for T cell recognition.

Because Ser^{711} is a putative phosphorylation site (*26*) and phosphorylation-dephosphorylation is a signal for proteins to be targeted for degradation and relocalization (*27–29*), we evaluated the effect of the mutation in CDC27 on Ag processing. Wild-type and mutated full-length (intact) CDC27 were transfected into 293IMDR4 cells. The CD4$^+$ TIL1359 recognized the mutated CDC27 (pcDNA-CDC27m) transfected cells, but not those with wild-type CDC27 (pcDNA-CDC27w) (Fig. 3B). To examine possible alterations of cellular relocalization of protein that conferred access of the mutated CDC27 protein to the MHC class II endosomal Ag presentation pathway instead of being targeted directly to the nucleus and centrosome (*18*), we constructed a fusion gene containing an Ii targeting sequence fused in-frame to the wild-type CDC27 cDNA. Transfection of pIi-CDC27w into the 293IMDR4 cells conferred recognition by CD4$^+$ TIL1359 comparable to pIi-CDC27m, which contained the Ii targeting sequence and mutated CDC27

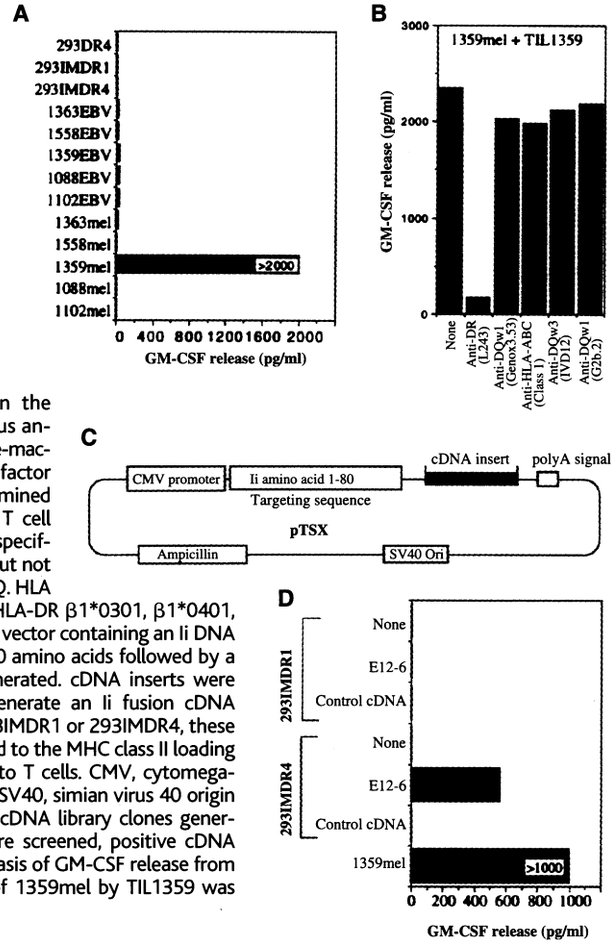

Fig. 1. Identification of a mutated CDC27 as a tumor Ag recognized by CD4$^+$ TIL1359. (**A**) Specific recognition of 1359mel by CD4$^+$ TIL1359. TIL1359 recognized the autologous 1359mel but did not recognize autologous 1359EBV, other EBV-B cell lines and allogeneic melanoma cell lines, nor HEK 293-derived cell lines. Both 1088mel and 1102mel share DRβ1*0401 with 1359mel. (**B**) HLA restriction of T cell recognition. CD4$^+$ TIL1359 cells were cocultured with autologous 1359mel cells in the presence or absence of various antibodies to MHC. Granulocyte-macrophage colony-stimulating factor (GM-CSF) release was determined after an 18-hour incubation. T cell recognition of 1359mel was specifically blocked by an anti-DR, but not by anti–MHC class I or anti-DQ. HLA genotyping of 1359mel was HLA-DR β1*0301, β1*0401, β3*0101, and β4*0101. (**C**) A vector containing an Ii DNA fragment encoding the first 80 amino acids followed by a multiple cloning site was generated. cDNA inserts were cloned into the vector to generate an Ii fusion cDNA library. After expression in 293IMDR1 or 293IMDR4, these Ii fusion proteins were targeted to the MHC class II loading compartment and presented to T cells. CMV, cytomegalovirus; polyA, polyadenylate; SV40, simian virus 40 origin locus. (**D**) After 1.6 × 10^5 Ii-cDNA library clones generated from 1359mel RNA were screened, positive cDNA pools were identified on the basis of GM-CSF release from CD4$^+$ TIL1359. Recognition of 1359mel by TIL1359 was used as a positive control.

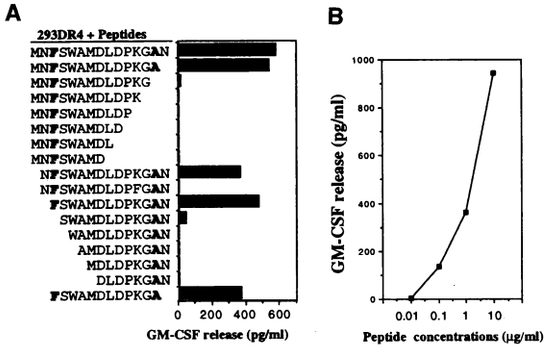

Fig. 2. Identification of epitopes recognized by CD4$^+$ TIL1359. (**A**) Characterization of CDC27 peptides recognized by CD4$^+$ TIL1359. A series of synthetic peptides were pulsed onto 293IMDR4 for 90 min. After three washes, T cells were added and tested for reactivity on the basis of GM-CSF release. A 12-amino acid peptide was defined as the minimal T cell epitope. (**B**) Peptide titration of T cell recognition. The 293DR4 cells were pulsed with different concentrations of the 12-amino acid CDC27 peptide for 90 min and washed with medium. T cells were added and incubated for 18 to 24 hours, and GM-CSF release was measured. TIL1359 recognized the 12–amino acid peptide at a concentration of 100 ng/ml.

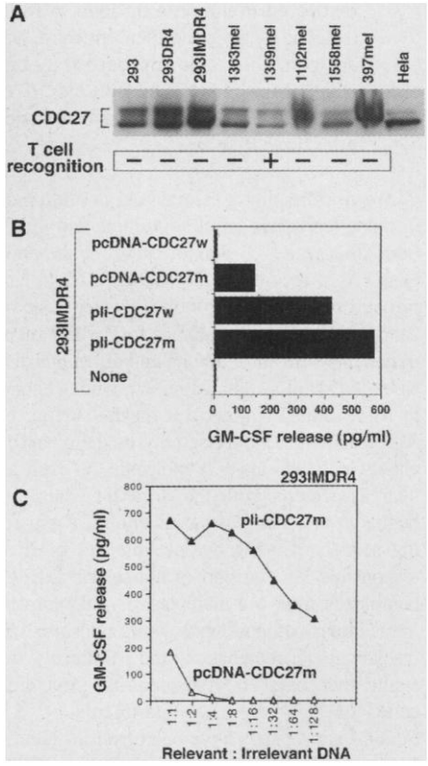

Fig. 3. Protein immunoblot analysis and T cell recognition. (**A**) Cell lystates were prepared and equal amounts of proteins were loaded and separated by SDS-PAGE (*25*). After being transferred to a membrane, two CDC27 bands (unphosphorylated and phosphorylated) were detected with monoclonal anti-CDC27 and the ECL system. CDC27 protein was recognized by CD4$^+$ T cells but was expressed at a lower level in the 1359mel line than in other cell lines tested. (**B**) 293IMDR4 cells were transfected with pcDNA-CDC27w (wild type), pcDNA-CDC27m (mutant), and their respective Ii fusion constructs pIi-CDC27w and pIi-CDC27m. T cell recognition was evaluated on the basis of GM-CSF release after coculture of CD4$^+$ T cells with transfectants for 18 to 24 hours. The wild-type CDC27 was recognized by CD4$^+$ T cells only when targeted by the Ii sequence. (**C**) Recognition of 293IMDR4 transfected with pIi-CDC27m (Ii-fused CDC27m) and pcDNA-CDC27m (without Ii fusion) by CD4$^+$ TIL1359 at different relevant:irrelevant cDNA ratios.

(Fig. 3B). Thus, the efficient targeting or relocalization of CDC27 to the MHC class II Ag presentation pathway was necessary and sufficient for Ag presentation to TIL1359.

Although the mutated CDC27 (pcDNA-CDC27m) could be recognized by $CD4^+$ TIL1359 when transfected into 293IMDR4, the reactivity was relatively weak compared with the Ii-fused mutated CDC27 (pIi-CDC27m) (Fig. 3B). Titration experiments demonstrated that $CD4^+$ TIL1359 could recognize the Ii-fused mutated CDC27 at dilutions of greater than 1:128 but failed to recognize the mutated CDC27 without Ii fusion at any dilutions greater than 1:2 (Fig. 3C). These results suggested that the Ii fusion significantly increased the efficiency of Ag processing and presentation through the MHC class II pathway and thus the sensitivity of T cell recognition.

We next examined whether the $Ser^{711}\rightarrow$Leu mutation affected the intracellular relocalization of CDC27 with an antibody to CDC27 and found that CDC27 was predominantly located in the cytoplasmic compartments of 1359mel cells (Fig. 4). In contrast, CDC27 in 1102mel and HEK 293 cells was mainly located in the nucleus, and only a small proportion in the cytoplasm (Fig. 4). This supported the hypothesis that the $Ser^{711}\rightarrow$Leu mutation affected relocalization of the CDC27 protein, which led to the generation of an epitope for T cell recognition in the MHC class II pathway.

To determine whether this genetic approach could be used to identify other Ags presented on MHC class II molecules and recognized by $CD4^+$ T cells, we studied $CD4^+$ TIL1558 cells derived from melanoma patient 1558. These $CD4^+$ TIL1558 could recognize an autologous tumor cell line (1558mel), but not autologous EBV-B cells (158EBV), nor other MHC class II–positive EBV-B and tumor cell lines (Fig. 5A). $CD4^+$ TIL1558 recognized both whole (class II^+) 1558 tumor cells and cell lysates of 1558mel pulsed on MHC-matched EBV-B cells and restricted by HLA-DRβ1*0101. The Ag recognized by TIL1558 is a mutated Ag identified with a biochemical approach (*7*). An Ii-cDNA fusion library was prepared from 1558mel RNA, 5.0×10^4 cDNA clones expressed in 293IMDR1 cells were screened with TIL1558, and positive pools were identified. After the second screening, 10 positive cDNA clones were isolated. The $CD4^+$ TIL1558 recognized 293IMDR1 transfected with cDNA clones 1 to 10 but not with a control cDNA (Fig. 5B). DNA sequencing analysis indicated that all 10 positive clones contained a mutation (C→T) at nucleotide position 450 in triosephosphate isomerase (TPI) (*30*). This mutation resulted in the substitution of isoleucine (ATT) for threonine (ACT) at amino acid position 28 and was identical to the previously identified mutation (*7*). The mutation was present in the 1558mel tumor cells but not in 1558EBV-B cells (*7, 13*). Transfection experiments indicated that $CD4^+$ TIL1558 recognized 293IMDR1 transfected with the mutated full-length TPI gene but not with the wild-type TPI gene, nor 293IMDR4 transfected with either the mutated or wild-type TPI (Fig. 5C), suggesting that the endogenously expressed mutated TPI protein can be processed and presented by DR1 molecules to $CD4^+$ TIL1558. In this case, the mutation itself constituted a T cell epitope recognized by $CD4^+$ TIL1558 (Fig. 5C) (*7*). Thus, two independent approaches identified the same mutated Ag recognized by $CD4^+$ TIL1558, further validating this genetic approach for the identification of MHC class II–restricted Ags.

We have described a general method for the screening of cDNA libraries to identify MHC class II–restricted Ags recognized by $CD4^+$ T cells. This approach was used to identify an Ag that could not be identified by a biochemical approach because tumor lysates pulsed exogenously onto Ag-presenting cells were not processed and presented efficiently for T cell recognition. Therefore, this genetic approach is generally applicable to the identification of autoantigens, mutated and xenogeneic Ags recognized by $CD4^+$ T cells derived from patients with cancer, autoimmune diseases, or infectious diseases, and may be useful for the development of preventive and therapeutic vaccines.

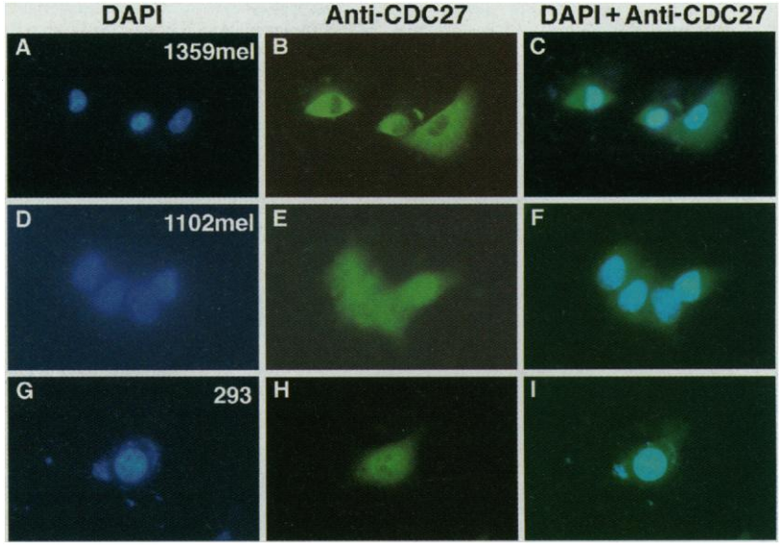

Fig. 4. Intracellular staining of CDC27 in 1359mel, 1102mel, and HEK 293 cells. The 1359mel, 1102mel, and HEK 293 cells were fixed with 1% paraformaldehyde (PFA) and then permeabilized. Anti-CDC27 was used as a primary antibody followed by incubation with a fluorescein isothiocyanate–conjugated anti-mouse antibody. DAPI (4′,6′-diamidino-2-phenylindole) stainings in (**A**), (**D**), and (**G**) were used to visualize the nucleus. The same set of cells stained with anti-CDC27 (**B**, **E**, and **H**) are indicated. The overlay of DAPI and anti-CDC27 staining is shown in (**C**), (**F**), and (**I**). CDC27 in 1359mel was predominantly located in the cytoplasm, whereas CDC27 in 1102mel and HEK 293 cells was stained mainly in the nucleus.

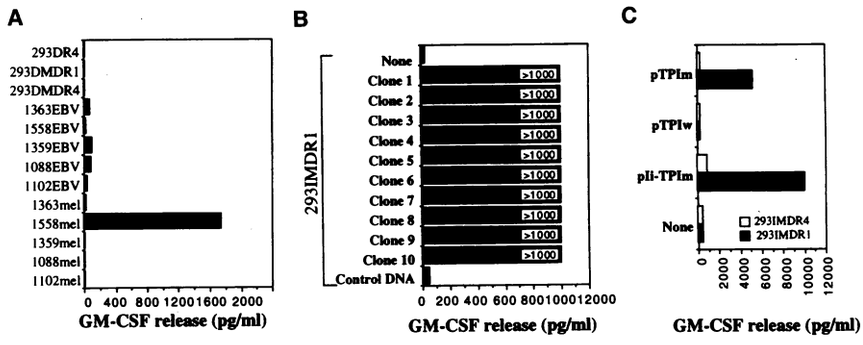

Fig. 5. Characterization of $CD4^+$ TIL1558 and identification of mutated triosephosphate isomerase (TPI) as a DR1-restricted Ag. (**A**) Specific recognition of 1558mel by $CD4^+$ TIL1558. 1363mel shared DRβ1*0101 with 1558mel. (**B**) After screening 5×10^4 Ii-cDNA library clones generated from 1558mel RNA, positive cDNA pools were identified on the basis of GM-CSF release from $CD4^+$ TIL1558. (**C**) Transfection of 293IMDR1 with the mutated plasmids pTPIm and pIi-TPIm conferred T cell recognition. However, transfection of 293IMDR1 with wild-type plasmid pTPIw and 293IMDR4 with either wild-type or mutated plasmids failed to stimulate GM-CSF release from $CD4^+$ TIL1558.

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9. HEK 293 cells were transfected with plasmids encoding DRα, DRβ, Ii, DMA, and DMB individually. Transfectants were selected in media containing G418, hygromycin, and puromycin. Expression of human leukocyte AG DR (HLA-DR) was evaluated by fluorescence-activated cell sorting analysis with either monoclonal antibodies to HLA-DR1 or HLA-DR4 (One Lambda, Canoga Park, CA). Expression of DMA, DMB, and Ii was detected by protein immunoblot with antibodies to DMA (anti-DMA), anti-DMB, and anti-Ii.
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11. The constructs pHG810, pIHG812, pIHG813, and pIHG814 were made as follows. The Ii fragments (amino acids 1 to 60, 1 to 80, and 1 to 108) were generated by polymerase chain reaction amplification with specific sets of primers and cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, San Diego, CA) to produce the pI60, pI80, and pI108 plasmids, respectively. A 400–base pair DNA fragment containing the HA(307–319) epitope was amplified from a plasmid encoding an influenza virus HA and was inserted into pI60, pI80, and pI108 to generate pIH60, pIH80, and pIH108, respectively. A DNA fragment encoding enhanced green fluorescence protein (EGFP) from pEGFP-N3 (Clontech, Palo Alto, CA) was then fused in-frame to the COOH-terminus of the IH fusion constructs. The resultant plasmids were named pIHG812, pIHG813, and pIHG814. pHG810 was constructed in the same way except for omission of the Ii fragment.
12. Supplementary material is available at www.science.org/feature/data/987217.shl
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15. Total RNAs were extracted from 1558mel and 1359mel with Trizol reagent from GIBCO BRL (Gaithersburg, MD). Polyadenylated RNAs were purified from total RNA by using polyATract System (Promega, Madison, WI) and converted to cDNA with a cDNA construction kit (GIBCO BRL) with an oligo-dT primer. The cDNA inserts were cloned into the expression vector pTSX. The cDNA libraries were electroporated into DH10B bacteria cells (GIBCO BRL). Plasmid DNA pools were prepared from bacteria, each consisting of about 100 cDNA clones.
16. The cDNA library from 1359mel was divided into cDNA subpools, each of which consisted of about 100 cDNA clones (*15*). A total of 1.6×10^5 cDNA clones were screened by expression in 293IMDR4 cells (*17*). Two positive pools were identified in the first screening. Individual DNA was prepared from about 800 *Escherichia coli* colonies transformed with the positive-pool DNA, and six positive cDNA clones were isolated by repeating the screening procedure.
17. DNA transfection with GM-CSF assays were performed as previously described [R. F. Wang, P. F. Robbins, Y. Kawakami, X. Q. Kang, S. A. Rosenberg, *J. Exp. Med.* **181**, 799 (1995); R.-F. Wang *et al.*, *ibid.* **184**, 2207 (1996); R.-F. Wang *et al.*, *J. Immunol.* **161**, 3596 (1998)].
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22. Peptides were synthesized by a solid-phase method with an automatic peptide synthesizer (Model AMS 422, Gilson, Worthington, OH). The masses of some peptides were confirmed by mass spectrometry, and peptides were purified by high-pressure liquid chromatography as needed to obtain purities greater than 98%.
23. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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25. Cell lysates were prepared by lysis buffer. Equal amounts of total protein were loaded for each sample and separated on a 4 to 16% SDS-polyacrylamide gel by electrophoresis (SDS-PAGE). The proteins were then blotted onto a nitrocellulose membrane and incubated with a murine antibody to human CDC27 (Transduction Lab) or a rabbit polyclonal antibody to human CDC27. After incubation with an anti-mouse or anti-rabbit immunoglobulin G conjugated with horseradish peroxidase, protein was detected with the ECL system (Amersham).
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BRCA1 Inhibition of Estrogen Receptor Signaling in Transfected Cells

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Mutations of the breast cancer susceptibility gene *BRCA1* confer increased risk for breast, ovarian, and prostatic cancers, but it is not clear why the mutations are associated with these particular tumor types. In transient transfection assays, *BRCA1* was found to inhibit signaling by the ligand-activated estrogen receptor (ER-α) through the estrogen-responsive enhancer element and to block the transcriptional activation function AF-2 of ER-α. These results raise the possibility that wild-type BRCA1 suppresses estrogen-dependent transcriptional pathways related to mammary epithelial cell proliferation and that loss of this ability contributes to tumorigenesis.

Germ line mutations of the *BRCA1* gene (17q21) (*1*) account for 40% to 50% of hereditary breast cancers and confer increased risk for ovarian and prostatic cancers (*2*). The *BRCA1* gene encodes an 1863-amino acid protein with a highly conserved NH_2-terminal RING finger domain and a COOH-terminal acidic transcriptional activation domain (*1, 3*). The product of the *BRCA1* gene is a 220-kD nuclear phosphoprotein (*4*) that has been implicated in regulation of cell proliferation, cell cycle progression (*4, 5*), apoptosis induction (*6, 7*), and DNA repair and recombination (*7, 8*). These functions of *BRCA1* have been observed in various human epithelial cancer cell types and mouse fibroblasts and do not explain the association of *BRCA1* mutations with specific tumor types, such as breast cancer. Estrogen stimulation of mammary epithelia is thought to be a major factor in promoting development of

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