A novel human gene (PLU-1) has been identified which shows a highly restricted expression in normal adult tissues but which is consistently expressed in breast cancers. A fragment of the PLU-1 cDNA was identified by differentially screening a fetal brain library with cDNAs prepared from ce-1 cells (a human mammary epithelial cell line overexpressing c-ErbB2) treated or untreated with the antibody D5, which inhibits c-ErbB2 phosphorylation. Clones covering the full cDNA sequence of 6.4 kilobases were isolated from a breast cancer cDNA library. Although expression of PLU-1 in ce-1 cells is regulated by signaling from c-ErbB2, the gene is expressed in all the breast cancer cell lines examined, in cells cultured from primary breast cancers, and in the invasive and in situ components of primary breast cancers. Translation of the open reading frame predicts a protein of 1544 amino acids, which contains three PHD/LAP motifs, a specific DNA-binding domain found in a Drosophila protein (dri) and novel domains showing extensive homology with other human and non human gene products. Transient transfection of cell lines with MYC-tagged PLU-1 showed the protein to be localized in the nucleus and associated with discrete foci. The presence of the dri motif and PHD/LAP fingers together with the clear nuclear localization and consistent expression in breast cancers, suggest a role for PLU-1 in regulating gene expression in breast cancers.

The genetic changes that occur in cancer, whether these be mutations or alterations in levels of gene expression, become evident as changes in the phenotype of a specific cell type. In characterizing these phenotypic changes in malignancy, it is therefore important to work with the appropriate cells or cell lines. Breast cancers show the phenotype of the luminal epithelial cell (1), which can be cultured from milk, and cell lines have been developed from these milk cells which retain the luminal phenotype (2). One of these cell lines (MTSV1–7) has been used to look at the effect of overexpression of various oncogenes and proto-oncogenes on the behavioral properties of this cell type (3, 4).

Overexpression of the c-ErbB2 receptor has been observed in a proportion of breast cancers and found to correlate with a poor prognosis (5), making signaling from this receptor an important parameter for investigation. To study the function of c-ErbB2 in human mammary epithelial cells, the receptor was overexpressed in MTSV1–7 cells to produce the ce-1 cell line (3).

Unlike other receptors in the ErbB family, the c-ErbB2 homodimer has no known ligand, although c-ErbB2 can function as a heterodimeric receptor for the heregulin family of ligands with c-ErbB3 or c-ErbB4 or for EGF with c-ErbB1 (6–9). Signaling from c-ErbB2 in overexpressing cells is, however, thought to be constitutive, operating through autophosphorylation of the homodimer, which forms because of overexpression (10).

To look for genes whose expression is reversibly regulated by c-ErbB2 signaling as a homodimer, we have down-regulated c-ErbB2 phosphorylation using an antibody that has been shown to inhibit signaling from the receptor in breast cancer cell lines (11). In the humanized form (12), the antibody is under investigation in the clinic for the treatment of breast cancer (13). cDNAs prepared from ce-1 cells, treated or untreated with the antibody D5, were used to differentially screen filters from a fetal brain library using a computerized analysis (14), and a partial clone was isolated representing a novel sequence. Clones covering the full-length sequence (6.4 kb) of the novel PLU-1 gene were subsequently isolated by screening a breast cancer cDNA library.
Comparison of the PLU-1 protein sequence with sequences predicted from other human and non-human genes, identified three cysteine-rich PDH/LAP domains, one of which appears to be highly conserved throughout evolution, suggesting an important function hitherto undefined. The PLU-1 gene product also contains a motif common to a family of sequence-specific DNA-binding proteins, exemplified by the product of the dead ringer Drosophila gene, dri (15). Another highly conserved domain in PLU-1 is a novel Trp/Tyr/Thr/Cys-rich region that is present in other human genes products and in proteins predicted from sequences in other species including Drosophila, Caenorhabditis elegans, yeast, and plants. C-terminal to this domain, and conserved with it in the same genes (with the exception of S. cerevisiae), is a small novel cysteine/histidine-rich motif.

Although the PLU-1 gene shows a highly restricted expression in normal adult tissues (being well expressed only in testsis), it is well expressed in primary breast cancers and breast cancer cell lines but not colon cancer cell lines. In view of its restricted expression, expression in breast cancers and breast cancer cell lines, and the presence of the dri motif and PDH/LAP domains, PLU-1 is a candidate protein for regulating gene expression in breast cancer cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Cell Lines—MTSV-1, ce-1, T47D, and ZR75 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS (Life Technologies, Inc.) and 0.3 μg/ml gentamicin. This medium was supplemented with 5 μg/ml hydrocortisone (Sigma) and 10 μg/ml insulin (Sigma) for MTSV-1 cell lines and ce-1. For ce-1 cells, the selectable marker G418 (Life Technologies, Inc.) was also added at a concentration of 500 μg/ml. The SKBR-3 and MCF-7 cells were grown in RPMI containing 3.7% bicarbonate, 10% FCS (Life Technologies, Inc.), and 0.3 μg/ml gentamicin. The same medium with added insulin was used for MCF-7. The BT20 cell line was maintained in MEMBic with 15% FCS plus insulin and gentamicin.

**Culture of Primary Breast Carcinomas**—Two samples of invasive breast carcinomas (numbers 4 and 9) provided by the Hedley Atkins/Imperial Cancer Research Fund Breast Pathology Group at Guy’s hospital were cut into 1–2-mm² sections and digested with 20 ml of collagenase (Sigma) at 450 units/ml in DMEM medium with 10% FCS overnight on a rotary shaker. After washing with DMEM in decreasing concentrations of FCS (10, 5, and 2%), the cells were grown in 1.05 mM 1-methyl-3-isobutylxanthine (Sigma), 10 mM 3-isobutyl-1-methylxanthine (Sigma), 10 μg/ml of EGF (Amersham), 10 μg/ml of insulin (Sigma), and 10 ng/ml of bovine pituitary derived IGF-1 (Sigma, St. Louis, MO) for 48 h. Then 1 ml of medium with 10% FCS was added, and the cells were grown for 8 days. The cell pellet was then digested with 5 ml of 0.25% dispase (Boehringer Mannheim, Germany) and 0.25 M EDTA. The remaining cell mass was digested further with 3 ml of 0.05% trypsin/EDTA and 0.1% Pluronic (Sigma). After washing with 10 ml of RPMI containing 10% FCS, 1 ml of fresh medium was added, followed by 0.1 ml of insulin. After 1 day, 1 ml of fresh medium was added again. At the end of this period, the supernatant was discarded, and 1 ml of fresh medium with 10% FCS, 10 μg/ml of EGF (Amersham), 10 μg/ml of insulin (Sigma), and 10 ng/ml of IGF-1 (Sigma, St. Louis, MO) was added. After an 8-h incubation, the cells were trypsinized and replated in fresh medium.

**Isolation of cDNA Coding for the Novel PLU-1 Gene**

**Isolation of the First Partial Clone—**ce-1 cells were grown to approximately 50% confluence and then grown for 48 h in the presence or absence of 50 ng/ml of the 45D mAb (kindly provided by Dr. M. Sliwkowski, Genentech), which inhibits phosphorylation of c-ErbB-2 on tyrosine residues (12). Poly(A)⁺ RNA was isolated from total RNA from the treated and untreated cells using oligo(dT) chromatography according to the poly(A) Quik kit (Stratagene), then converted to cDNA using the Superscript II reverse transcriptase (Life Technologies, Inc.). The cDNAs were subsequently used as probes labeled with [α-³²P]dCTP by random priming.

Filters carrying 10⁶ clones from a cDNA library made from human fetal brain were hybridized with the above labeled probes. The labeling was evaluated by computerized analysis with a phosphorimager. Different clones were screened and expression was confirmed by Northern blot of the ce-1 cells. Analysis of seven clones demonstrated a novel sequence in clone 253G2, which gave a weaker signal with the probe from the 45D-treated cells.

**Isolation of Clones Covering the Full PLU-1 Gene**—For isolation of further sequences of the gene containing the 253G2 sequences, three cDNA libraries were used, namely a ZR75 phage library, a Jurkat plasmid library, and a testis phage library. The cDNA library from the human breast carcinoma cell line ZR75 was oligo(dT)-primed, and cDNA sequences were cloned into the uni-ZAP XR vector (Stratagene) with a XhoI site at the 5’ end and EcoRI site at the 5’ end (17). 10⁶ plaques from the ZR75 library were screened initially using a fragment of approximately 253G2 sequence and subsequently with 5’ sequence obtained from the longer clones. Three consecutive screenings were performed and 22, 27, and 12 plaques picked, respectively, from the original plates. The plaques containing the largest clones with most 5’ sequence were determined by touchdown and seminested PCR on the original plaques. Plaques were then purified by secondary and tertiary screens and pBS-SK⁺ – plasmids obtained by in vivo excision.

Since the 3’ end of the gene was not obtained in the three screens of the ZR75 library, a Jurkat cDNA library was screened. This library was prepared by priming cDNA from the human T-leukemia cell line J6 with random hexamers (18). The whole library was screened by PCR using a sequence from the ZR75 clone containing the most 5’ sequence. The PCR product was purified using a JET-sorb DNA Extraction kit (GENOMED GmbH, Germany) and sequenced. 450 bp of new 5’ sequence was thus obtained and used as a probe for a fourth screen of the ZR75 library from which the clone 1.2 was isolated. 280 bp of sequence was covered by only one clone (between consensus sequence 665–937). This piece of the sequence was further confirmed by screening a human testis 5’-STRETCH PLUS cDNA Library from Clontech and isolating clones covering the sequence. Sequencing was performed using an ABI PRISM 377 DNA Sequencer, Perkin-Elmer.

The entire sequence was obtained from at least two individual clones covering the same region, and both were sequenced in each direction. Analysis of the consensus cDNA sequence revealed a single long ORF of 4632 nucleotides, starting at position 90 and ending with a TAA termination codon at 4725. The sequence encodes a 1544-amino acid protein with a predicted size of 170 kDa. The 3’-untranslated region of 1589 nucleotides contains a terminal poly(A) region of 65 As.

**Assembly of Full-length PLU-1 cDNA**

**Construction of the Full-length PLU-1 cDNA—**Three overlapping clones (ZR75 1.2, 3.1, and 14) containing unique restriction enzyme sites were used for construction of the full-length cDNA. The most 5’ clone (clone 1.2) in the Bluescript plasmid was cut with BgII at base 466 and with XhoI at the 3’ end cloning site, leaving the 5’ 466 bp of the PLU-1 sequence in the plasmid vector. The second clone (3.2) was digested with BgIII/Aur II and the 2435-bp fragment isolated. The third clone (14) was cut with AvrII/XhoI and the 3476-bp fragment comprising the rest of the 3’ sequence was separated. The 3’ fragments were then joined together in one reaction with T4 DNA ligase. The recombination fragments were sequenced over the join regions, and the final construct with a 6.4-kb insert was referred to as pBS-SK⁺ – plu1. The procedure is shown diagrammatically in Fig. 1A.

**Construction of a Tagged PLU-1 Mammalian Expression Vector—**Based on the analysis of the restriction enzymes in the sequence and the amino acid coding sequence, the mammalian expression vector pcDNA 3.1 (+/MYC-His A (Invitrogen) with a C-terminal MYC-His tag driven by the cytomegalovirus promoter was selected for constructing the tagged gene. A 3’ PLU-1 coding fragment (632 bp) was generated by PCR, where, at the 3’ end, the TAA stop codon was replaced to give an XhoI site flanked by a HindIII site. (The HindIII site at the 3’ end allowed the cloning into the pcDNA vector, while the XhoI site allowed the whole PLU-1 sequence to be retrieved if required.) The 3’ sequence on the coding strand generated by the pB HindIII antisense primer is aligned below with the wild type sequence.

| GAC GCA CCA AGC AGA AAG | TAA AAA CAC AAA AAC AGA (wild type) |
|---------------------------|-------------------------------------|
| GAC GCA CCA AGC AGA AAG | CTC GAG GAG CTT AAC AG (wild type) |

XhoI/HindIII sequence

The 5’ primer included an NcoI site to link the PCR fragment to the rest of the PLU-1 sequence, which was excised as a 4106-bp XbaI/NcoI fragment from the pBS-SK⁺ – PLU-1 construct. The PCR product (cut with NcoI and HindIII), the XbaI/NcoI fragment, and the pcDNA 3.1 MYC-HisA vector, linearized with XhoI and HindIII, were then ligated in one reaction. The recombinant clones were sequenced over the joins and PCR regions and the final construct with a 4.781-kb insert is referred as plu1-ORF/MYC-His A.
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**Fig. 1. Diagrammatic representation of the PLU-1 cDNA showing the three clones used to assemble the full-length cDNA (A) and homologies with other non-human genes (B).** A, boxed number 1 domains: dPLU-1 dri motif (ZR75 library with 235G2. Clone 3.1 was isolated by screening the library with a probe from the 5′ end of clone 14. Clone 1.2 was obtained by screening the ZR75 library with the 5′ sequence obtained from the Jurkat library by PCR (see "Experimental Procedures"). B, specific motifs in PLU-1 dri motif (II), aa 75–190 (also see Fig. 8); novel Trp/Tyr/Phe/Cys motif (I), aa 366–637; novel Cys/His motif (III), aa 692–725. PDI/LAP domains: boxed number 1, aa 313–361; boxed number 2, aa 1179–1221; boxed number 3, aa 1486–1534. •, nuclear localization signals: I, aa 227–234; II, aa 286–302; III, aa 1103–1120; IV, aa 1400–1417; V, aa 1434–1440.

**Electroporation**—The expression of the recombinant protein with the tagged PLU-1-ORF/MYC-His A construct was first checked by transient expression of COS cells. The cells were grown to 70% confluence, trypsinized, washed with PBS, and 5 × 10⁶ cells resuspended in 1 ml of PBS with 20 μg of DNA either from the MYC-His A construct or the empty vector as control. The cells were electroperforated with a Gene Pulser (Bio-Rad) using 250 microfarads at 450 V and then resuspended in 30 ml of growth medium and plated on 9-cm dishes and glass coverslips for Western blot analysis and immunostaining, respectively.

**Calcium Phosphate-mediated Transfection**—Breast cancer cell lines (T47D, MCF-7, BT.20, ZR.75, and the HT1080 cell line) were grown on 3-cm dishes to approximately 60% confluence, trypsinized, washed with PBS, and 5 × 10⁶ cells resuspended in 1 ml of PBS were used for detecting PLU-1 mRNA and the 4.4-kb HindIII fragment of the pSV2Er2B2 for c-ErbB2 mRNA. To assess the efficiency of loading and transfer of the RNA, the membranes were reprobed for GAPDH expression.

**Immunofluorescent Staining**

Cells on coverslips or 3-cm dishes were washed with PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton for 5 min. After blocking with 20% FCS/PBS for 30 min, cells were incubated with the 9E10 mAb to the MYC tag (10 μg/ml) and then with fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig 1:50 (Dako).

**Western Blot Analysis**

The level of inhibition of tyrosine phosphorylation of the c-ErbB2 gene product with Ab 4D5, and the expression of the MYC-tagged PLU-1 gene product from transiently transfected COS cells was assessed by subjecting 100 μg of total lysates to immunoblot analysis with the respective antibodies. Confluent ce-1 cells (treated or untreated with 4D5 Ab) in 9-cm tissue culture dishes were washed three times with cold PBS containing 1 mM sodium orthovanadate and then lysed with 1 ml of lysis buffer (3). For detection of the recombinant MYC-tagged PLU-1 gene product, the

**Northern Analysis of RNA from Cell Lines and Strains**

Total cellular RNA from the cell lines or cultures of primary breast cancers was isolated according to the method of Chomczynski and Sacchi (19). The total cellular RNA of the colon cancer cells was a kind gift from Helga Durbin. 20 μg of RNA from each cell type was denatured in 1 × MOPS, 0.66 M formaldehyde, and 50% (v/v) formamide and subsequently size-fractionated on a 1.2% formaldehyde-agarose gel. After clarification of the lysates by centrifugation at 15,000 × g for 10 min at 4 °C, the protein concentration of the lysates was estimated using the Bio-Rad protein assay kit. Samples were then electrophoretically separated on a 5% stacking/7.5% running SDS-polyacrylamide gel electrophoresis, transferred to Hybond-C membrane (Amersham). Immunoblots were blocked with 5% bovine serum albumin or 5% skimmed milk, 0.1% Tween 20 in PBS for 2 h, probed with anti-phosphotyrosine mAb PY20, 1:100 (Upstate Biotechnology) or 1 μg/ml anti-MYC mAb, 9E10, for 2 h. The immune complexes were detected with 125I-labeled sheep anti-mouse Ig 0.5 μCi/ml (Amersham) for PY20 or peroxidase-conjugated rabbit anti-mouse Ig 1:2000 (Dako) for 1 h. Bands were detected using an enhanced chemiluminescence detection kit (Amersham).

**A 15635**

**B**

**C**

**PLU-1**

CEZK933.4

DMAC1714

MMCMCX

ATF2013.10

ATF19113.11

SCYRIR13C

SCYER169W

**Fig. 1. Diagrammatic representation of the PLU-1 cDNA showing the three clones used to assemble the full-length cDNA (A) and homologies with other non-human genes (B). A, boxed number 1 domains: dPLU-1 dri motif (ZR75 library with 235G2. Clone 3.1 was isolated by screening the library with a probe from the 5′ end of clone 14. Clone 1.2 was obtained by screening the ZR75 library with the 5′ sequence obtained from the Jurkat library by PCR (see "Experimental Procedures"). B, specific motifs in PLU-1 dri motif (II), aa 75–190 (also see Fig. 8); novel Trp/Tyr/Phe/Cys motif (I), aa 366–637; novel Cys/His motif (III), aa 692–725. PDI/LAP domains: boxed number 1, aa 313–361; boxed number 2, aa 1179–1221; boxed number 3, aa 1486–1534. •, nuclear localization signals: I, aa 227–234; II, aa 286–302; III, aa 1103–1120; IV, aa 1400–1417; V, aa 1434–1440.**
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**FIG. 2.** Full-length protein sequence of PLU-1 deduced from the cDNA sequence showing homology to other human and non-human gene products. For accession numbers, see Table I. A, multiple sequence alignment of PLU-1 and related human sequences. The sequences were aligned using Clustal X (35) with homology shading according to the PLU-1 sequence (black indicates identity to PLU-1, and gray indicates similarity with PLU-1 or identity/similarity within the other sequences). Gaps were adjusted manually where appropriate. The дри or dead ringer domain (see Fig. 8) is indicated by a light gray dotted line above the sequence alignment. The three PHD domains are indicated with thick black lines (below the alignment). The novel Cys/His domain is indicated with a narrow black line (above the alignment). Nuclear import signals are indicated with gray lines (above the alignment). The RB-binding domain in RBP2 is boxed (1374–1378). B, multiple sequence alignment.
of PLU-1 and related non-human sequences. The sequences were aligned using Clustal X (35) with homology shading according to the PLU-1 sequence (black indicates identity to PLU-1, and gray indicates similarity with PLU-1 or identity and similarity within the other sequences). Gaps were adjusted manually where appropriate. Conserved domains in PLU-1 are labeled according to A. It is notable that the C. elegans sequence is of similar length to PLU-1 and contains all of the identified conserved domains, suggesting that it may represent a PLU-1 homologue. There are two putative nuclear receptor binding motifs in PLU-1 (Leu-X-X-Leu-Leu; Ref. 36), which are conserved only in the mammalian PLU-1 related sequences (residues 890–894 and 961–965).
FIG. 2—continued

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In Situ Hybridization of Sections of Breast Tissues and Tumors

Blocks of formalin-fixed, paraffin-embedded tissues and tumors were obtained from the archives of the Imperial Cancer Research Fund Breast Pathology Group at Guys Hospital. For specific localization of the PLU-1 mRNA by in situ hybridization, an antisense riboprobe synthesized from the 253G2 clone was prepared. The plasmid was linearized with EcoRI and a labeled 1074-bp antisense probe synthesized with T7 polymerase and 35S-UTP (800 Ci/mmol, Amersham). This sequence at the 3' end of the PLU-1 mRNA showed minimal homology to other human genes. The presence of hybridizable mRNA in all compartments of the tissues studies was established in near serial sections using an antisense probe generated with SP6 RNA polymerase and DraI-linearized phBA-10, prepared by subcloning a 450-bp fragment of a human β-actin cDNA into SP73 (20). The methods for pretreatment, hybridization, washing, and dipping of slides in Ilford K5 for autoradiography have been modified (20) from those described previously (21). Films were exposed for 7 and 15 days for the PLU-1 probe, and for 7 days for the β-actin probe, before developing in Kodak D19 and counterstaining with Giemsa. Sections were examined under conventional or reflected light dark-field conditions (Olympus BH2 with epi-illumination) that allowed individual autoradiographic silver grains to be seen as bright objects on a dark background.

Fluorescence in Situ Hybridization of PLU-1 for Chromosomal Localization

30 metaphase spreads prepared from phytohemagglutinin-stimulated normal human lymphocytes by standard techniques were analyzed. Before hybridization the slides were denatured in 70% formamide and 0.2 M SSC at 73 °C for 3 min, washed in 0.2 M SSC, and dehydrated through an ethanol series of cold 70%, 95%, and absolute ethanol. Probe DNA (either 253G2 or the full-length sequence from pBS-SK+ PLU-1) was biotinylated using the Bionick kit (Life Technologies, Inc.). 500 ng of labeled probe was mixed with 5 μg of Cot-1 DNA (Life Technologies, Inc.), precipitated, resuspended in 11 μg of hybridization mix, denatured at 85 °C for 5 min, and allowed to preanneal at 37 °C for 30 min. After preannealing, the probe was applied to a denatured slide and hybridized at 37 °C overnight.

Slides were washed in 50% formamide, 2 × SSC, pH 7.0 at 42 °C, followed by 1 × SSC at 60 °C. Blocking solution (3% bovine serum albumin, 4 × SSC, and 0.1% Tween 20) was applied and slides incu-
bated at 37 °C for 30 min. After incubation, avidin-fluorescein isothiocyanate (diluted in 1% bovine serum albumin, 4 × SSC, 0.1% Tween 20) was applied and slides incubated at 37 °C for 40 min. Slides were washed in 4 × SSC, 0.1% Tween 20 at 42 °C and counterstained with DAPI (4,6-diamidino-2-phenylindole, 200 ng/ml), followed by 2 min in 2 × SSC. Slides were mounted in Citifluor and images captured using a Photometrics KAF 1400–50 CCD camera attached to a Zeiss Axioskop epifluorescence microscope. Separate images of probe signals and DAPI banding patterns were pseudocolored and merged using SmartCapture software (Vysis, Inc., Chicago, IL). In all the spreads a signal was observed on both copies of chromosome 1 band 1q32.1. No other consistent signal was observed.

RESULTS

Isolation of the PLU-1 Gene—The MTSV1–7 cell line was derived by immortalization of luminal epithelial cells cultured from human milk (2), and the ce-1 cell line was developed by transfection of MTSV1–7 with c-ErbB2 cDNA (3). To look for genes whose expression is regulated by signals generated through c-ErbB2, phosphorylation of the receptor was down-regulated by treatment with the 4D5 antibody for 48 h. The cDNAs, prepared from mRNA from ce-1 cells treated or untreated with antibody, were then used as labeled probes to differentially screen a fetal brain cDNA library. The clone 253G2, which showed a weaker signal with cDNA from the 4D5-treated cells, was identified and the insert sequenced. Using 5′ sequences from the 253G2 clone, further clones covering and extending the 253G2 sequence were isolated from a cDNA library prepared from a breast cancer cell line (ZR75). Further screens of the breast cancer library were required to obtain the full sequence, and three overlapping clones were assembled (as described under “Experimental Procedures”) to give a full-length cDNA of 6394 bp, of which 4632 represent the coding region (see Fig. 1A).

The PLU-1 sequence shows homology with a number of other human gene products which are shown optimally aligned in Fig. 2 and summarized in Table I. These include the retinoblastoma-binding protein RBP-2 (22, 23), the X-linked but not inactivated gene product XE169 (24), and related gene product on the Y chromosome (25) as well as an unknown gene product KIAA (26). Among these, RBP-2 has the most homology with...
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PLU-1 and is of similar length, whereas XE169 (hssmcX in Fig. 2), HSSMCY, and KIAA are shorter at their C termini, respectively. Interestingly, the RB binding motif LXCKE, which is found in RBP2 at the 3’ end, is not present in PLU-1 or the other related sequences. PLU-1 also exhibits strong homology with sequences found in other organisms (see Figs. 1B and 2B and Table I). These include the XE169 mouse homologues and gene products predicted from sequences in C. elegans (27), Drosophila, Saccharomyces cerevisiae, and Arabidopsis that have not yet been functionally defined (for accession numbers see Table I). The most extensive homology is seen with the C. elegans and mouse sequences, but certain domains are conserved in most of the sequences (see below).

The PLU-1 Gene Is Consistently Expressed in Breast Cancers—The original sequences isolated in clone 253G2 were used to examine expression of PLU-1 mRNA by Northern analysis. The 253G2 clone contains some translated sequence together with untranslated sequence (3633–5559 bp), all of which show little homology with the other human genes shown in Fig. 2A, and therefore this probe should detect only PLU-1 mRNA. Fig. 3 shows that the level of expression of PLU-1 mRNA in ce-1 cells decreases after treatment with the 4D5 antibody, which strongly inhibits phosphorylation of c-ErbB2. Expression of PLU-1 mRNA was low in the nonmalignant MTSV1–7 cell line, but all the breast cancer cell lines examined showed expression (Fig. 4A). Although signaling from c-ErbB2 clearly regulates the expression of PLU-1 (Fig. 3), the level of expression in the cell lines does not correlate with the level of expression of c-ErbB2 mRNA. However up-regulation of PLU-1 is not seen in all cancer derived cell lines as expression was not detected in a series of colon cancer derived lines as shown in Fig. 4B.

Fig. 4B also shows that PLU-1 mRNA is detected in cultures of primary breast cancers. Although it is difficult to culture malignant cells from primary breast cancers, we were able to culture one tumor (tumor 4) for two passages and prepare mRNA (lane 8). Another culture from a primary breast cancer (tumor 9), after transfection with the BCL-2 gene, could be cultured for several passages. Fig. 4B shows that again PLU-1 mRNA could be detected in malignant cells and not in normal cells (lane 9). Significantly, transfection of BCL-2 into the MTSV1–7 cell line did not induce expression of PLU-1 (data not shown), indicating that expression of the gene correlates with malignancy, not with expression of BCL-2.

To evaluate expression of PLU-1 in primary breast cancers more fully, in situ hybridization was performed using a riboprobe prepared from the 253G2 clone and sections of breast cancers and benign lesions (see “Experimental Procedures”). Fifteen malignant tumors were examined (four ductal grade 1, four ductal grade 2, four ductal grade 3, and three lobular carcinomas). In all the ductal carcinomas and in two of the three lobular carcinomas, the invasive component showed the highest level of expression. In situ components also showed strong labeling with the 253G2 probe, while benign components of the carcinomas were negative or weakly positive except when closely bordering the invasive component, when the labeling became stronger. Three fibroadenomas and two lactating adenomas showed only a weak signal with the PLU-1 probe. Fig. 5 shows examples of labeling of invasive, in situ, and benign components of a grade 1 and a grade 3 ductal carcinoma. Although the numbers are small, the results suggest that PLU-1 expression is up-regulated in breast cancers but not in benign lesions with the highest expression being seen in the invasive component.
Restricted Expression of PLU-1 in Normal Adult Tissues—To assess the expression of PLU-1 in normal adult tissue, Northern blots of mRNA from a range of tissues were probed with the 253G2 probe. Fig. 6 shows that the only tissue showing a high expression of PLU-1 is testis, although low levels of expression of mRNA were detectable in placenta, ovary, and tonsil. A restricted analysis of normal tissues with in situ hybridization confirmed the limited expression of PLU-1 and expression in testis (in Sertoli cells) was also confirmed. Apparently expression of PLU-1 is highly restricted in normal adults, which distinguishes it from the homologous RBP-2 and human E169a genes, reported to be ubiquitously expressed (23, 24). The chromosomal location of PLU-1 also distinguishes it from the homologous genes as it is located on chromosome 1q32.1 as shown in Fig. 7.

Specific Domains in PLU-1—Within the PLU-1 sequence, there are several previously identified motifs, which are found in proteins having a function in transcription, and these are indicated in Fig. 2. PHD/LAP motifs are zinc-binding domains, which are thought to mediate protein-protein interactions and are commonly found in transcription factors (28, 29). PLU-1 contains two well defined PHD/LAP fingers (residues 313–361 and residues 1179–1221) and an imperfect PHD/LAP finger (residues 1486–1534). The protein also contains a sequence-specific DNA-binding motif (residues 75–190) first identified in the Drosophila dead ringer gene (15). The motif from drr has been shown to bind the same DNA sequence in vitro as the engrailed protein (which contains a classic homeodomain DNA-binding motif), even though drr and engrailed show no sequence homology (15). The dead ringer or drr DNA-binding motif is also found in several unrelated gene products (Fig. 8), including the SWI protein, which is known to be part of a complex that promotes activator binding to nucleosomal DNA (30). Moreover, one of the genes in this family, jumonji, is a gene known to be crucial for neuronal development in the mouse (31). Thus, members of this family may be important in regulating gene expression that is associated with particular cell phenotypes. All the PLU-1-related human sequences contain both the drr motif and two of the three PHD/LAP fingers with RBP-2 containing all three PHD/LAP domains (Fig. 2A and Table I). As shown in Figs. 1B and 2B, the mouse, C. elegans, and Drosophila gene products also contain the first PHD domain and the drr motif (see Table I).

In addition to the domains already functionally defined, two other novel domains are present in PLU-1, which are conserved in the sequences of the related gene products. A striking region of high sequence conservation is found between residues 366–637, which is rich in aromatic amino acids and cysteines (~14% Trp/Tyr/Phe). This region is well conserved within all the PLU-1-related sequences from human genes and from other species (Table I and Figs. 1B and 2). Immediately C-terminal to this region is a small conserved Cys/His region (residues 692–725) of unknown identity. From the cross-species alignment (Fig. 2B), the following sequence pattern suggestive of a novel potential metal-binding domain can be obtained: Cys-X2-Cys-X3-Cys-X2-Cys/His-X4-7-Cys-X2-His-X5-3-Cys-X2-Cys. Sequence searches for these two novel motifs show no significant matches other than those shown in Fig. 2. It is notable that the plant-related sequences only show homology to the above two regions in PLU-1 (residues 366–773), suggesting that the Trp/Tyr/Pherich sequence and the novel Cys/His motif may be associated to form a potentially functional domain, conserved in otherwise unrelated gene products.

Cellular Localization of the PLU-1 Gene Product—There are five putative nuclear localization signals in the PLU-1 sequence, which are indicated in Fig. 2A and Table I. The presence of these sequences, as well as domains indicative of a role in transcription, suggest that PLU-1 is a nuclear protein. To determine the intracellular location of the protein, PLU-1 was tagged with a MYC epitope recognized by the antibody 9E10 and transiently expressed in COS cells. Western blot analysis of extracts of the transfected cells using the anti-MYC antibody detected a single band of the expected size (170 kDa, Fig. 9).

Immunohistochemical staining of the transfected cells 3 days after transfection showed unambiguously that the protein was localized to the nucleus, but not the nucleolus (Fig. 10, A–C). Fig. 10D, representing a composite image from confocal microscopy, shows that the staining of the tagged gene product is clearly associated with discrete foci in some of the cells. Similar patterns of staining were obtained in transient transfections of other cell lines (breast cancer cell lines T47D, MCF7, ZR-75, BT20, HT1080, MTSV1–7, and two nonepithelial cell lines HT1080 and HB96, data not shown).

All attempts to obtain permanent transfectants with the tagged PLU-1 cDNA in the cell lines indicated above were unsuccessful. After the addition of the selectable marker (G418), some cells survived, but all lost expression of PLU-1. A total of 45 clones were selected from transfections of COS cells, HT1080, MTSV1–7, T47D, MCF7, and ZR75, and in no case was PLU-1 expression maintained. Fig. 10 (E and F) illustrates...
A Novel Gene (PLU-1) Up-regulated in Breast Cancer

We have described here the initial characterization of a novel human gene, PLU-1, which shows restricted expression in normal adult tissues, but which is consistently expressed in primary breast cancers and in breast cancer cell lines. Although the gene was identified as being up-regulated by signaling from the c-ErbB2 receptor, expression is not limited to breast cancer cells expressing high levels of c-ErbB2. However, the expression of PLU-1 is associated with the invasive and in situ components in primary breast cancers and is expressed only weakly or not at all in benign tumors. Thus PLU-1 expression is closely associated with the malignant phenotype of breast cancer.

A comparison of the predicted amino acid sequence of the PLU-1 gene product with other sequences in the data base reveals striking homologies with a number of sequences from human, flies, worms, yeast, and plants, which suggest a role for PLU-1 in transcription. Within the PLU-1 sequence, there are three well defined PHD/LAP motifs (28, 29), which are conserved cysteine-rich sequences thought to bind divalent metals to form autonomously folded domains, similar to other known Cys-rich domains (32). They are also often found in proteins that appear to be involved in chromatin-mediated transcriptional regulation (33). PLU-1 and related sequences (except for the yeast and plant gene products) also contain a dri motif, which was first reported in the dead ringer protein from Drosophila and has been shown to mediate sequence specific DNA binding (15). This would suggest a role for PLU-1 as a specific DNA-binding protein, which is consistent with the nuclear localization of the tagged protein in transfected cells.

The functions of some of the other dri-containing proteins have also been studied extensively. Of particular interest is the yeast SWI-1 gene with human homologues (34), which form part of a large multiprotein complex involved in regulating transcription by modifying nucleosome conformation (28, 29). In this context, immunohistochemical staining of the PLU-1 protein in transfected cells showed the protein to be localized in discrete foci in some cells. The mouse jumonji gene is also a member of the dri family and appears to have a tissue specific function, since it is crucial for neuronal development. Thus, the dri motif, the nuclear localization, and the presence of PHD domains all suggest a role for PLU-1 in transcriptional control, and the restricted expression implies an important function in breast cancer cells.

One striking region of high homology between PLU-1 and other gene products from plants and yeast to humans is the Trp/Tyr/Phe Cys-motif region. In all except the yeast genes, this region is followed 3’ by a cysteine-rich motif with a conserved histidine (Cys-X2-Cys-X2-Cys/Cys/His-X2-Cys/His-X2-Cys-Cys-X2-Cys). The function of this combined sequence has not been clarified, and in the case of the nonmammalian genes, the sequences are contained within longer regions that have merely been reported as contiguous sequences. However, the high degree of cross-species sequence conservation strongly suggests a potential function for this region/domain of fundamental importance. It is notable that in the putative plant proteins, sequences flanking this region are unrelated to PLU-1 (and the other related sequences see Fig. 1B), which supports the suggestion that this domain encompasses an evolutionary conserved functional module.

The failure to develop permanent transfectants of seven different cell lines with the tagged PLU-1 gene was surprising, and suggested that very high expression levels are not compatible with viability. It is a well established fact that while human mammary epithelial cells can be cultured from normal tissue and from benign tumors, malignant cells from primary breast cancers rarely survive culture, with most of the cell lines being derived from late metastases. It is possible that the increased expression of PLU-1 seen in breast cancers relates to their initial poor survival in culture. This aspect requires further study, in particular to eliminate the possibility of artifacts in the failure to obtain permanent transfectants. Clearly the development of specific antibodies to allow a more detailed evaluation of PLU-1 expression and to locate endogeneous...
PLU-1 protein is an important priority.

There are multiple sequences in the PLU-1 protein that are predicted to be Class I epitopes for several HLA alleles, some of which are in regions with minimal homology to other human genes. Together with the restricted expression of the gene in adult tissues, this suggests that the gene product could be a target antigen for immunotherapy of breast cancer where the high level of expression in testis would not present a problem in female patients. With the availability of the PLU-1 cDNA it will be possible to examine this possibility using both human PBLs and mouse models.

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