

BCAR1, a Human Homologue of the Adapter Protein p130Cas, and Antiestrogen Resistance in Breast Cancer Cells

Arend Brinkman, Silvia van der Flier, Elisabeth M. Kok, Lambert C. J. Dorssers

Background: Treatment of breast cancer with the antiestrogen tamoxifen is effective in approximately one half of the patients with estrogen receptor-positive disease, but tumors recur frequently because of the development of metastases that are resistant to tamoxifen. We have previously shown that mutagenesis of human estrogen-dependent ZR-75-1 breast cancer cells by insertion of a defective retrovirus genome caused the cells to become antiestrogen resistant. In this study, we isolated and characterized the crucial gene at the breast cancer antiestrogen resistance 1 (BCAR1) locus. **Methods/Results:** Transfer of the BCAR1 locus from retrovirus-mutated, antiestrogen-resistant cells to estrogen-dependent ZR-75-1 cells by cell fusion conferred an antiestrogen-resistant phenotype on the recipient cells. The complete coding sequence of BCAR1 was isolated by use of exon-trapping and complementary DNA (cDNA) library screening. Sequence analysis of human BCAR1 cDNA predicted a protein of 870 amino acids that was strongly homologous to rat p130Cas-adapter protein. Genomic analysis revealed that BCAR1 consists of seven exons and is located at chromosome 16q23.1. BCAR1 transcripts were detected in multiple human tissues and were similar in size to transcripts produced by retrovirus-mutated ZR-75-1 cells. Transfection of BCAR1 cDNA into ZR-75-1 cells again resulted in sustained cell proliferation in the presence of antiestrogens, confirming that BCAR1 was the responsible gene in the locus. **Conclusions:** Overexpression of the BCAR1 gene confers antiestrogen resistance on human ZR-75-1 breast cancer cells. Overexpression of BCAR1 in retrovirus-mutated cells appears to result from activation of the gene's promoter. The isolation and characterization of this gene open new avenues to elucidating mechanisms by which the growth of human breast cancer becomes independent of estrogen. [J Natl Cancer Inst 2000;92:112-20]

The steroid hormone estradiol plays a pivotal role in breast cancer initiation and cell proliferation (1,2). Estradiol and its receptor participate in a multiprotein complex that acts as a transcription regulator for various genes (3-6). In approximately two thirds of all breast tumors, the estrogen receptor (ER) is present and functionally active (7-9). The presence of the ER in breast carcinoma cells and their responsiveness to estrogens is clinically exploited by the administration of antagonists of estrogens or antiestrogens (10-12). On binding, antiestrogens induce an aberrant conformation of the ER, resulting in an inactive transcription complex (13). As a consequence, the antiestrogen-receptor complex may bind DNA but can no longer modulate the expression of its target genes (14). This inhibition of the estrogen response pathway (15) may block cellular proliferation (16-18) and arrest tumor growth (19,20).

During the last 2 decades, antiestrogens, particularly tamoxi-

fen, have proved to be effective in the treatment of hormone-responsive breast cancer (21-23). Adjuvant treatment with tamoxifen reduces tumor recurrence and increases survival of patients with ER-positive breast cancer (24,25). In metastatic breast cancer, tamoxifen leads to objective response in nearly one half of patients with ER-positive primary tumors (26). Resistance to antiestrogens, however, is a serious obstacle in the management of breast cancer. About 40% of ER-positive tumors fail to respond to antiestrogen therapy (intrinsic resistance) (27), whereas eventually most, if not all, breast tumors that initially respond to antiestrogens develop resistant metastases (acquired resistance).

The mechanisms underlying intrinsic or acquired antiestrogen resistance of breast tumors are still poorly understood. Altered pharmacology of the antiestrogens (19,28,29), modification of the ER structure and function (30-32), and changes in the interactions between tumor cells and their environment (paracrine interactions) (33) have been proposed to contribute to the development of antiestrogen resistance. In addition, genetic or epigenetic changes in the tumor cells that promote the development of antiestrogen resistance have been postulated (34,35). Although each of these possibilities may account for or contribute to the resistant phenotype in individual patients, none so far has been shown to explain antiestrogen resistance in a majority of patients. It is likely that progression to antiestrogen resistance is a multifactorial process.

We attempted to identify the genetic factors that may lead to antiestrogen resistance. Therefore, we applied retroviral-insertion mutagenesis to the human estrogen-dependent breast cancer cell line ZR-75-1. We demonstrated that random integration of a defective murine retrovirus in the genome of ZR-75-1 cells transformed the cells from an estrogen-dependent to a tamoxifen-resistant phenotype (36). Mapping of common integration sites and cell fusion-mediated gene transfer experiments so far have identified three independent loci that are involved in antiestrogen resistance (BCAR1, BCAR2, and BCAR3) (37). Here we report the isolation and characterization of the target gene of the BCAR1 locus and its functional involvement in antiestrogen resistance *in vitro*.

MATERIALS AND METHODS

Cell Lines

The human breast carcinoma cell line ZR-75-1 and derivatives thereof were cultured as described elsewhere (36,38). Hybrid cell lines and BCAR1-

Affiliation of authors: Department of Pathology/Division of Molecular Biology, Josephine Nefkens Institute, University Hospital Rotterdam, The Netherlands.

Correspondence to: Arend Brinkman, Ph.D., Department of Pathology/Division of Molecular Biology, Josephine Nefkens Institute, University Hospital Rotterdam, Rm. Be 435a, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands (e-mail: brinkman@bidh.azr.nl).

See "Notes" following "References."

© Oxford University Press

transfected cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated bovine calf serum and 1 nM 17 β -estradiol. For BCAR1-transfected cell lines, 500 μ g/mL of the neomycin analogue G418 (Life Technologies, B.V., Breda, The Netherlands) was added to the culture medium.

The tamoxifen-resistant cell line XI-13, with two copies of the defective LN retroviral genome (36,39), one located within the BCAR1 locus, was lethally γ irradiated (40 Gy) and subsequently fused to hygromycin B-resistant ZR-75-1 (ZH3D7) cells with polyethylene glycol as described previously (37). G418 plus hygromycin selection was performed in estradiol-containing culture medium (37).

Molecular Biology Techniques

A human genomic cosmid library in pJB8 (40) was obtained from W. J. M. van de Ven (University of Leuven, Leuven, Belgium) and screened by use of standard protocols (41).

Exon trapping was performed according to the manufacturer's instructions (Life Technologies, Inc). In short, genomic DNA of the cosmid clones HC26 and HC34 was partially digested with the enzyme *Mbo*I. Fragments of approximately 5–10 kilobases (kb) (average, 7.5 kb) were isolated and cloned in the exon-trap vector pSPL3. Batches of pooled purified plasmid DNA from five clones each were transfected into the simian kidney cell line (COS-1) (41). After transient expression, RNA was isolated and complementary DNA (cDNA) was synthesized. Following two rounds of polymerase chain reaction (PCR), the trapped sequences were analyzed, cloned, and sequenced.

RNA was isolated from the somatic hybrid D4E5 (containing the BCAR1 locus) and cultured for 2 days with 4-hydroxy-tamoxifen by use of the CsCl-guanidine thiocyanate procedure (41). Poly(A) RNA was selected with the use of the PolyATtract mRNA Isolation System (Promega Corp., Madison, WI) and converted into cDNA (Zap Express cDNA and cloning kit; Stratagene Cloning Systems, La Jolla, CA) as recommended by the manufacturer. A cDNA library of approximately 2×10^6 phages was created and screened. Twenty-one phage clones hybridizing with a BCAR1 exon-trap probe were isolated and sequenced by use of standard procedures (41). DNA and protein sequence analyses and alignments were performed with the use of DNASIS (Hitachi Software Engineering America Ltd., Brisbane, CA), Blast (www.ncbi.nlm.gov/blast), and Clustalw (www2.ebi.ac.uk/clustalw) software. The BCAR1 sequence has been assigned GenBank Accession No. AJ 242987.

Northern blots were prepared by use of 1% agarose-formaldehyde gels as described previously (41). Blots were hybridized with random-primer-labeled probes for BCAR1, the estrogen-regulated PS2 gene, and the glyceraldehyde-3-phosphate dehydrogenase gene for loading control as described (42,43).

Mapping of the BCAR1 locus was performed on genomic DNA from a panel of 30 somatic cell hybrids obtained from D. F. Callen (Women's and Children's Hospital, Adelaide, Australia). PCR was performed with the locus-specific primers 1140 5'-CCCCACATACCCAGCACA-3' and 1142 5'-CCCAGTCTCTC-CTTATTCA-3'. The amplified product was verified by hybridization with the BCAR1 locus-specific probe 18/1 (36).

Expression Constructs and Transfection of BCAR1 in ZR-75-1 Cells

The full-length BCAR1 cDNA was inserted into the *Eco*RI-*Xho*I site of the long terminal repeat-promoted pLXSN expression vector (39) and into a modified version of the episomal vector LZRS-IRES-Neo (LZRSpBMN-IRES) (44). In the latter vector, the BCAR1 cDNA and the neomycin resistance gene (to confer selectability) were separated by an IRES sequence, so that both genes were under control of a single long terminal repeat (LTR) promoter and transcribed as a polycistronic messenger.

Stable transfectants were generated with the pLXSN/BCAR1 construct by the use of calcium phosphate precipitation (41). Transfection of ZR-75-1 cells with the episomal LZRSpBMN-IRES/BCAR1 vector construct was performed by use of Lipofectin reagent (Life Technologies, Inc.), according to the manufacturer's instructions. Transfectants were selected for neomycin resistance (500 μ g of G418/mL) in estradiol-containing medium.

Proliferation Assay

Somatic hybrid cells, BCAR1, and mock-transfected cells were grown in culture medium supplemented with 1 nM estradiol. Cells were harvested at 80% confluence by trypsinization. The cells (0.7×10^6) were seeded in 25-cm² flasks, in triplicate, and grown in the presence of either 1 μ M OH-tamoxifen or 100 nM

of the pure antiestrogen ICI 182,780 (Zeneca Pharma, Ridderkerk, The Netherlands). After 4–6 days, the cells were again trypsinized, counted using a Coulter Z1 cell counter (Coulter Electronics Ltd, Luton, U.K.), and reseeded in triplicate as above. To follow the proliferation of the cells, the procedure was repeated at several time points and for each cell line. In case of limited recovery of cells at the end of the assay period, only two flasks were reseeded or the culture was terminated. Proliferation was determined as the fold multiplication (mean of at least two flasks) relative to day 0.

Drug-sensitivity tests on ZR-75-1-derived cell lines were performed in complete medium containing estradiol in 96-well plates. Five thousand cells were plated with threefold dilutions (four wells each) of the drug. After 9 days of culture in the presence of the drug, viable cells were measured by use of the MTT assay as described (38).

Bcar1 Protein Detection

BCAR1 and mock-transfected cells were trypsinized, rinsed with phosphate-buffered saline, sonicated for 10 seconds, and lysed for 10 minutes at 100 °C in 1% sodium dodecyl sulfate (SDS) and 10 mM Tris-HCl (pH 7.5). The protein concentration of the crude lysates was determined (BCATM, Protein assay reagent; Pierce Chemical Co., Rockford, IL). Equal amounts (3.3 μ g) of protein were used for SDS-polyacrylamide gel electrophoresis (PAGE) (8% acrylamide) and subjected to western blot analysis as described (45). Bcar1 was visualized with the mouse monoclonal antibody to rat p130Cas (Transductional Laboratories, Lexington, KY). In addition, polyclonal antibodies raised against the N-terminus and C-terminus of rat p130Cas (Cas N-17 and Cas C-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used to identify Bcar1 in an independent control experiment.

RESULTS

Transfer of the BCAR1 Locus by Cell Fusion

We have used retrovirus insertion-mediated mutagenesis on the human estrogen-dependent breast cancer cell line ZR-75-1 to generate 80 cell lines showing resistance to 4-hydroxy-tamoxifen (36). Integration of the defective murine retrovirus (LN) into the host cell genome could transform these cells to a tamoxifen-resistant phenotype. With the use of integration site-specific probes, we previously found a common integration locus for the retrovirus in a subset of this panel of cell lines. Four independent cell clones were identified by Southern blot analysis, with a viral integration in the same orientation at different positions within a 2.2-kb genomic region (36) (see also Fig. 2, A). We termed this locus BCAR1, for breast cancer antiestrogen resistance 1 locus—i.e., the first BCAR locus identified.

To establish whether retroviral integration in the BCAR1 locus induces tamoxifen resistance in ZR-75-1 cells, we transferred the genomic region encompassing the BCAR1 locus from tamoxifen-resistant cells into estrogen-dependent ZR-75-1 cells by cell fusion. XI-13 cells carry two copies of the defective retroviral genome, one copy of which is located within the BCAR1 locus. On lethal γ irradiation, the cells were fused to hygromycin B-resistant ZR-75-1 cells (ZH3D7) with polyethylene glycol as described previously (37). The neomycin resistance gene in the integrated retrovirus allowed the isolation of somatic cell hybrids by dual selection with G418 plus hygromycin in estradiol-containing culture medium. Twelve cell hybrids containing either one of the integration loci in the ZR-75-1 background were rescued and analyzed. Southern blotting analysis by use of a specific probe for the neomycin resistance gene revealed that five hybrids had retained the BCAR1 integration locus (a 15-kb *Bgl*II fragment), whereas the remaining seven carried the other integration locus (a 10-kb *Bgl*II fragment). Both the virus-induced BCAR1 cell line XI-13 and the BCAR1 locus-containing cell hybrids derived therefrom (e.g., D4E5 and

D4E6) showed enhanced proliferation when cultured in the presence of the antiestrogen ICI 182,780 (Fig. 1, A). Hybrid cell lines carrying the other locus, as well as the ZH3D7 recipient cells, were growth inhibited. In the absence of antiestrogens, BCAR1 locus-containing hybrids also demonstrated increased proliferation compared with the control cell lines (Fig. 1, B). Note that the proliferation of most cell lines is more efficient in the absence of antiestrogen, which is likely because of some residual estrogens in the serum-containing medium. In the presence of antiestrogens, the ER pathway is more effectively blocked. The lack of the ER in XI-13 cells (36) can explain the comparable growth levels of these cells in the presence as well as in the absence of antiestrogens (Fig. 1). These fusion experiments demonstrated unambiguously that the BCAR1 locus induced antiestrogen-resistant proliferation of the somatic cell hybrids and that the BCAR1 phenotype is dominant.

Cloning of the BCAR1 Locus

A human cosmid library was screened with genomic probes that have been employed to identify the BCAR1 locus (36). The probes included a 500 base-pair (bp) *Apa*I-*Pst*I fragment (14B2) and a 450-bp *Sph*I fragment (18/1). Both probes reside at the center of the viral integration site in the BCAR1 locus (Fig. 2, A). Cosmids were isolated and analyzed by restriction mapping, resulting in a continuous contig of approximately 80 kb (Fig. 2, A). Unique probes derived from the 80-kb contig identified no further proviral integrations events in the 80 antiestrogen-resistant cell lines from our panel.

Identification of the BCAR1 Gene

Two of 10 overlapping cosmid clones covering the major part of the BCAR1 locus—HC26 and HC34—were selected for identification of transcripts by exon trapping. A total of 31 trapped sequences were recovered, of which 16 were related to known genes. Eight clones corresponded to exons 2–7 of the chymotrypsin gene (accession No. NM_001906), and the remaining

eight appeared to be homologous to sequences of the rat p130Cas gene (accession No. D29766) (46). Of the remaining 15 clones, two seemed to be generated because of cryptic splicing events in frequently occurring alu-type repetitive sequences in the BCAR1 locus, while the other clones did not show homology to any known sequences in available databases.

The trapped exons were hybridized to polyA messenger RNAs (mRNAs) isolated from the antiestrogen-resistant BCAR1 cell lines (XII-13, XI-14, and XI-13) and from the parental cell line ZR-75-1. The chymotrypsin gene sequences did not hybridize in a northern blot analysis, indicating that this gene is not expressed in our ZR-75-1-derived cell lines. In contrast, exon sequences that were homologous to the rat p130Cas gene hybridized to a single mRNA of 3.2 kb (Fig. 2, B). This transcript appeared to be increased in the antiestrogen-resistant cell lines but not in the parental cell line. As shown in Fig. 2, B, ZR-75-1 exhibits only low levels of an equally sized transcript. The differential expression of this transcript suggested that the rat p130Cas-homologous gene was the candidate breast cancer antiestrogen resistance gene, BCAR1. Conclusive evidence for its role in antiestrogen resistance required the isolation of the BCAR1 cDNA and its functional transfer to ZR-75-1 cells by transfection (*see below*).

Primary Structure of the BCAR1 Gene

A cDNA library from the antiestrogen-resistant BCAR1 cell hybrid D4E5 was screened with p130Cas homologous sequences retrieved from exon trapping. Twenty-one positive cDNA clones were identified and assembled by sequence alignment into a continuous cDNA of 3208 bp. The BCAR1 cDNA encloses an open-reading frame of 2610 nucleotides. At position 122, an initiation codon is flanked by sequences matching Kozak's criteria (47). The open-reading frame is flanked at the 3' end by a translation termination codon (TGA) at position 2732 and a 3' untranslated sequence of 468 nucleotides that contains multiple stop codons. A canonic polyadenylation site is

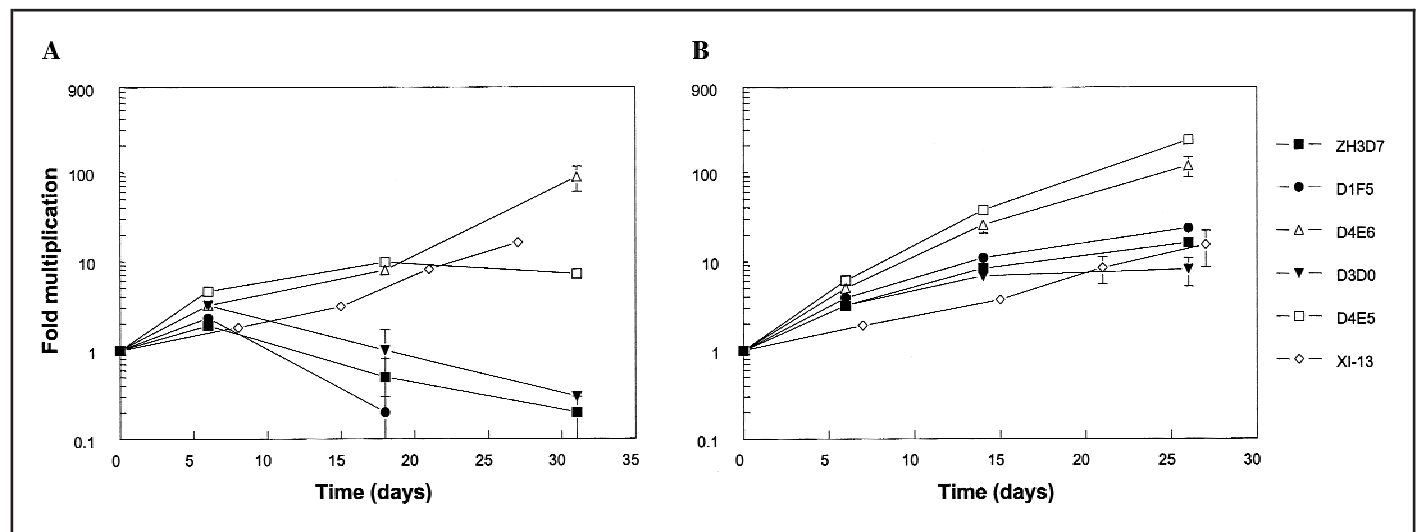


Fig 1. Antiestrogen resistance assay of somatic cell hybrids. Antiestrogen-sensitive recipient cells (ZH3D7), antiestrogen-resistant donor cells (XI-13), and somatic cell hybrids with (D4E5 and D4E6) and without (D1F5 and D3D0) BCAR1 locus were cultured in triplicate in serum-containing medium with 100 nM of ICI 182,780 (panel A) or without (panel B). At the times indicated, cells were harvested, counted, and reseeded in triplicate. The numbers of cells recov-

ered under antiestrogen at day 18 were insufficient to continue the culture of D1F5 and only allowed for reseeding in duplicate for ZH3D7 and D3D0 (panel A). Culture of the steadily growing XI-13 cells under antiestrogen was terminated at day 27 (panel A). Indicated is the fold multiplication (mean of at least two flasks \pm standard deviation) relative to day 0. Error bars were smaller than symbols in cases where they do not appear.

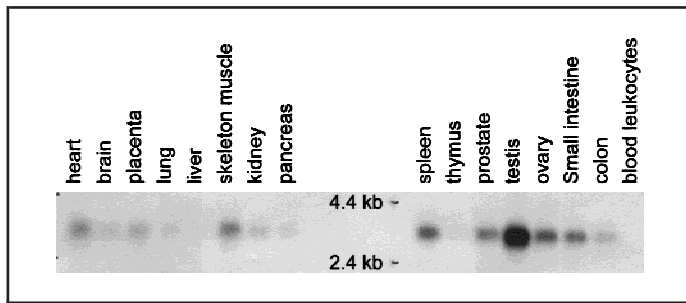


Fig. 4. Expression of BCAR1 in various human tissues. Commercially available northern blots containing polyA messenger RNA (2 μ g) of multiple human tissues (MTN blots; Clontech Laboratories, Inc, Palo Alto, CA) and checked for loading by the supplier were hybridized with a labeled BCAR1 total complementary DNA, washed until $0.3 \times$ standard saline citrate at 65°C for 30 minutes, and exposed to an x-ray film for 4 days at -70°C with an intensifying screen. Size markers are indicated.

antibody directed against rat p130Cas identified the BCAR1 gene product (Fig. 5, A) as well as polyclonal antibodies that are directed against either the N-terminus or the C-terminus of the rat p130Cas (data not shown).

Genomic Organization of BCAR1

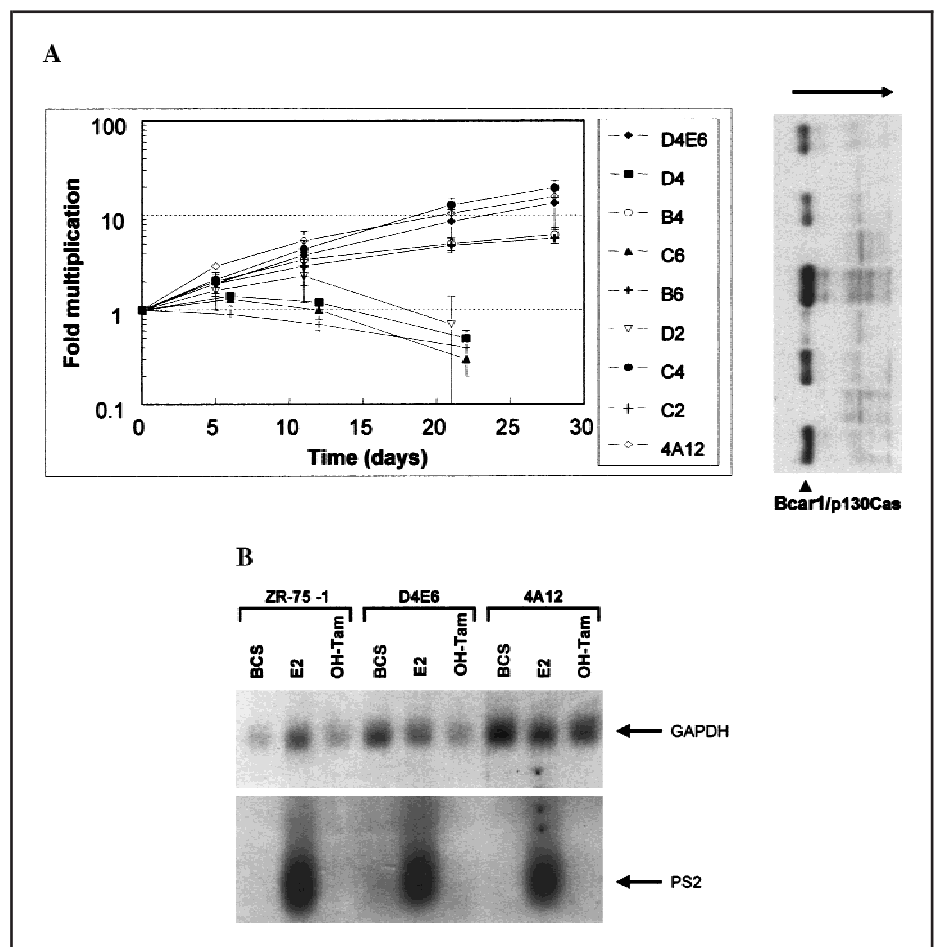
The genomic organization of the BCAR1 gene was delineated by use of the BCAR1 cDNA as a template. Restriction mapping and partial sequencing of cosmid clones HC26 and HC3 revealed that the transcribed region of the BCAR1 gene consists of

seven exons (I–VII) spanning approximately 25 kb of genomic DNA (Fig 2, B). The size of the exons varies from 76 to 1101 bp, and the size of the introns varies from 182 bp to approximately 8.5 kb. Sequencing through the exon–intron boundaries demonstrated that all splice junctions conform to the GT/AG rule for exon–intron junctions. Southern blot analysis showed that all restriction fragments from the cosmid clones that hybridized to BCAR1 cDNA were also present in genomic DNA. More important, the coding sequence of the BCAR1 gene was found to be identical to that of the cDNA, excluding cloning artifacts.

Exon 1 of BCAR1 contained the first 12 bp of the coding sequence. It is of interest that this position of the exon–intron boundary exactly matched the position where alternative splicing has been observed in rat as well as mouse p130Cas (46,49). Exons 2–6 all mapped within the coding sequence, and exon 7 contained the last 509 coding nucleotides and the complete 3' nontranslated region. The 5' flanking region of the BCAR1 gene had a high G + C content (80%) and lacks suppression of CpG dinucleotides, which is characteristic for CpG islands. Such a configuration, which is often found for housekeeping genes, is consistent with the ubiquitous expression of the BCAR1 gene (Fig. 4).

In situ hybridization with the complete cosmid HC26 suggested that the BCAR1 gene was located at human chromosome 16q22–23 (Van Aghoven T: personal communication). Primers specific for the integration region were used to analyze a panel of radiation hybrids containing fragments of human chromosome 16. PCR analysis on genomic DNA derived from these

Fig. 5. Characterization of BCAR1/p130Cas transfectants. **A) Left panel**—Growth curves of a representative selection of BCAR1-transfected cell lines grown in the presence of antiestrogen. Three flasks of each LXS/BCAR1/p130Cas-transfected cell line (4A12, B4, B6, and C4), the somatic cell hybrid (D4E6), and vector-only control cell lines (C2, C6, D2, and D4) were cultured in the presence of OH-tamoxifen (OH-Tam) (1 μ M). At indicated time points, three flasks were harvested, processed for cell counting, and reseeded. The number of cells recovered for C2 only allowed for reseeding in duplicate for the last time point. Indicated is the fold multiplication (mean of at least two flasks \pm standard deviation) relative to day 0 (see Fig. 1). **Right panel**—A typical western blot of cell lysates (3.3 μ g protein) separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (8%), transferred to nitrocellulose filters, and visualized with mouse monoclonal antibody to rat p130Cas. The blot contained cell lysates isolated from these cell lines at day 12/13. An **arrow** indicates the direction of migration in SDS–PAGE gels, and an **arrowhead** marks the position of Bcar1/p130Cas. Similar expression levels were observed on day 0 and on day 5/6. **B)** RNA was prepared from the indicated cell lines after culture in bovine calf serum-containing medium (BCS) supplemented with 1 nM of estradiol (E2) or 1 μ M of OH-Tam for 5 days. Approximately 5 μ g of RNA was blotted and hybridized with probes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PS2. Blots were washed until $0.3 \times$ standard saline citrate at 65°C for 30 minutes.



hybrid cell lines determined the position of the BCAR1 locus to 16q23.1. Hybrids lacking the region 16q23.2 to telomere (e.g., CY145) gave no BCAR1-specific product, whereas hybrids that retained the 16q23.1 region (e.g., CY116 and CY117) gave specific products (50). Evidence for this position of the BCAR1 gene was further supported by the concurrent trapping of exons of the chymotrypsin gene that had been assigned to chromosome 16q23.1 (50). Hybridization with chymotrypsin gene probes on the BCAR1 cosmid clones confirmed the position of the chymotrypsin gene 4–5 kb downstream of the BCAR1 gene (Fig. 2, A).

Considering the vast homology of the BCAR1 cDNA with rat and murine p130Cas sequences and the strict conservation of the first exon–intron boundary, we postulate that the BCAR1 gene is the human p130Cas homologue, and we will further refer to this gene as BCAR1/p130Cas.

Transfection of BCAR1/p130Cas cDNA and Estrogen Independence in ZR-75-1 Cells

To demonstrate that Bcar1/p130Cas is functionally involved in antiestrogen resistance, the full-length BCAR1/p130Cas cDNA was introduced in antiestrogen-sensitive ZR-75-1 cells by transfection. Five independent LXS/BCAR1/p130Cas transfectants were generated, carrying an intact transgene integrated in the host genome as determined by Southern blot analysis. Similarly, eight independent cell lines carrying the BCAR1/p130Cas cDNA in an episomal vector were established. In addition, more than 22 vector-control cell lines have been raised.

All transfected cell lines were generated in estrogen-containing medium and subsequently tested for proliferation in the presence of either 4-hydroxy-tamoxifen or the pure antiestrogen ICI 182,780. A typical example of such an assay with the use of 4-hydroxy-tamoxifen is shown in Fig. 5, A. Aliquots of the cultured cells have been used to measure the amount of Bcar1/p130Cas protein with the use of western blotting and immunodetection (Fig. 5, A). All cell lines exhibiting high expression of Bcar1/p130Cas (e.g., 4A12, B4, B6, and C4 and the somatic cell hybrid D4E6) showed net proliferation in the presence of 4-hydroxy-tamoxifen. In contrast, ZR-75-1 and all vector control cell lines (e.g., C2, C6, D2, and D4) expressed very low levels of or no Bcar1/p130Cas and failed to proliferate in the presence of the antiestrogen. Similar results were obtained in the assays by the use of the pure antiestrogen ICI 182,780 (data not shown). Of interest, Bcar1/p130Cas-transfected cells underwent a morphologic change when cultured in the presence of antiestrogens (data not shown). The cells displayed a typical flattened shape with multiple protrusions (similar to the virus-induced cell lines), which may be suggestive of a reorganization of the cytoskeleton. Further experiments with the use of immunofluorescence are needed to verify these observations. To investigate the possibility that Bcar1/p130Cas overexpression also generates resistance to other drugs, we performed cell survival assays by use of doxorubicin, 5-fluorouracil, and methotrexate. These pilot experiments showed no relation between sensitivity to these drugs and overexpression of Bcar1/p130Cas; i.e., comparable ID_{50} (dose showing 50% inhibition) values were obtained for overexpressing cell lines and cell lines without overexpression (data not shown).

Northern blotting analysis of Bcar1/p130Cas-transfected cells grown under antiestrogen selection revealed an impaired expression of the estrogen-regulated PS2 gene (43) similar to the pa-

rental cells (Fig. 5, B), suggesting that the proliferation of these cells was independent of ER function. Because integrin and growth factor-receptor pathways may signal through p130Cas to the mitogen-activated protein kinase (MAPK) pathways (51–54), we have performed preliminary experiments to monitor the activation state of various MAPKs in BCAR1/p130Cas-transfected cell lines. Specific antibodies directed against activated MAPKs (ERK, p38, and JNK) failed to document constitutive activation of these kinases in the presence of antiestrogens (not shown). Because of the slow growth and response kinetics of the transfected cells in antiestrogen-supplemented cultures, the levels of activated MAPK may have been below the threshold of detection in our tests.

DISCUSSION

The mechanisms underlying antiestrogen resistance in breast cancer are the subject of intensive study. Our working hypothesis was based on the assumption that genetic or epigenetic alterations in the tumor cells contribute to the failure of the therapy. To identify target genes, we have employed a model system of estrogen dependence resembling that seen in human breast cancer. In this article, we describe the cloning and characterization of the BCAR1 gene. On the basis of our findings, BCAR1 appears to be the human homologue of the rat and mouse p130Cas genes; their function is described below. Overexpression of Bcar1/p130Cas in human estrogen-dependent ZR-75-1 breast cancer cells is sufficient to drive cell proliferation in the presence of antiestrogens. No difference in structure or size of the BCAR1/p130Cas transcript was seen in the virus-infected cell lines. This suggests that overexpression of the BCAR1/p130Cas gene is achieved by increased activity of the BCAR1/p130Cas gene promoter, likely due to the presence of strong enhancer elements within the viral LTRs (55). This mechanism of transcription enhancement is different from the observed viral promoter-insertion activation of the BCAR3 gene that resulted in an altered BCAR3 transcript and protein (43). It is possible that such events are selected against, in the case of the BCAR1/p130Cas gene, to ensure an intact and functional protein.

Our experiments indicate that BCAR1/p130Cas-mediated antiestrogen resistance is not associated with resistance to other drugs relevant to the treatment of breast cancer. In addition, the ER does not play a crucial role in the resistant phenotype. Cell lines obtained after viral integration in the BCAR1 locus (e.g., XI-13) completely lack ER expression and function (36) and show comparable growth rates in the absence or presence of antiestrogens (Fig. 1). The somatic cell hybrids containing the BCAR1 locus and the BCAR1/p130Cas transfectants are estrogen responsive with respect to growth and regulation of PS2 expression. These variants exhibit antiestrogen-resistant cell proliferation but are not dependent on antiestrogen for growth (Fig. 1, B). These observations suggest a cell proliferation regulatory mechanism involving Bcar1/p130Cas, which bypasses the ER-regulated pathway.

The observation that the BCAR1 gene is highly homologous to the well-characterized rat and mouse p130Cas genes may help to understand the mechanism of antiestrogen-resistant breast cancer cell proliferation. Rat p130Cas has been identified as the major tyrosine-phosphorylated protein in v-Src- and v-Crk-transformed rat cells (46,56). Subsequent reports have implicated p130Cas in various processes in different cell types, including cell transformation (57,58), linking the extracellular

matrix with the actin cytoskeleton (59,60), integrin signaling (51,61–64), growth factor-receptor signaling (53,54,65–68), antigen-receptor signaling (69), cell migration and invasion (70–72), bacterial invasion (73,74), and essential cardiovascular development (58). This varied role of p130Cas may be explained by the presence of several protein–protein interaction domains (46,75), which is the hallmark of this novel class of adapter proteins. Family members are HEF1 (also designated CasL) (76,77) and Efs (also termed Sin) (78,79), which may have distinct functions (80). The SH3 domain, in particular, is well conserved between these family members and has been shown to interact with proline-rich target sequences from FAK (49), RAFTK (81), PTP-PEST (82), CAKbeta (83), and PTP1B (52). The central part of p130Cas contains multiple potential tyrosine–phosphorylation sites capable of interacting with SH2 domain-containing proteins like Crk (84,85), Src (48), and Nck (51). The C-terminal part of the p130Cas family members is well conserved but has not yet been implicated in a particular function in mammalian cells. Together, the numerous documented interactions and functions of p130Cas in various biologic processes and in different cell types suggest a dynamic role for p130Cas. The outcome of these complex interactions will depend on the availability of binding partners (including their activation status and affinity) and the specific process in that cell type.

So far, little information is available regarding the role of Bcar1/p130Cas in regulation of proliferation of breast epithelial cells. The observation that expression of Bcar1/p130Cas in primary breast cancer predicted disease prognosis and response to tamoxifen therapy (45) suggests a genuine role for Bcar1/p130Cas in growth control of breast cancer cells. Further elucidation of the pathway involved in Bcar1/p130Cas-mediated cell proliferation in breast cancer cells may identify *in vitro* key regulators and possibly novel clinical targets. The recognition that both BCAR1/p130Cas and BCAR3 (42) can control proliferation of breast cancer cells further supports the validity of our model system to identify breast cancer antiestrogen resistant genes.

REFERENCES

- Henderson BE, Ross R, Bernstein L. Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res* 1988;48:246–53.
- King RJ, William L. McGuire Memorial Symposium. Estrogen and progestin effects in human breast carcinogenesis. *Breast Cancer Res Treat* 1993;27:3–15.
- Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988;240:889–95.
- Truss M, Beato M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocr Rev* 1993;14:459–79.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. *Cell* 1995;83: 835–9.
- Beato M, Sanchez-Pacheco A. Interaction of steroid hormone receptors with the transcription initiation complex. *Endocr Rev* 1996;17:587–609.
- Horwitz KB, Wei LL, Sedlacek SM, D'Arville CN. Progesterone action and progesterone receptor structure in human breast cancer: a review. *Recent Prog Horm Res* 1985;41:249–316.
- Foekens JA, Rio MC, Seguin P, Van Putten WL, Fauque J, Nap M, et al. Prediction of relapse and survival in breast cancer patients by pS2 protein status. *Cancer Res* 1990;50:3832–7.
- Clarke RB, Howell A, Potten CS, Anderson E. Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res* 1997;57:4987–91.
- Jordan VC. Molecular mechanisms of antiestrogen action in breast cancer. *Breast Cancer Res Treat* 1994;31:41–52.
- Wolf DM, Fuqua SA. Mechanisms of action of antiestrogens. *Cancer Treat Rev* 1995;21:247–71.
- Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, McInerney EM, William L. McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. *Breast Cancer Res Treat* 1997;44: 23–38.
- Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature* 1997;389:753–8.
- Reese JC, Katzenellenbogen BS. Examination of the DNA-binding ability of ER in whole cells: implications for hormone-independent transactivation and the actions of antiestrogens. *Mol Cell Biol* 1992;12:4531–8.
- Parker MG. Action of “pure” antiestrogens in inhibiting ER action. *Breast Cancer Res Treat* 1993;26:131–7.
- Sutherland RL, Lee CS, Feldman RS, Musgrove EA. Regulation of breast cancer cell cycle progression by growth factors, steroids and steroid antagonists. *J Ster Biochem Mol Biol* 1992;41:315–21.
- Watts CK, Brady A, Sarcevic B, DeFazio A, Musgrove EA, Sutherland RL. Antiestrogen inhibition of cell cycle progression in breast cancer cells is associated with inhibition of cyclin-dependent kinase activity and decreased retinoblastoma protein phosphorylation. *Mol Endocrinol* 1995;9: 1804–13.
- Guvakova MA, Surmacz E. Tamoxifen interferes with the insulin-like growth factor I receptor (IGF-IR) signaling pathway in breast cancer cells. *Cancer Res* 1997;57:2606–10.
- Jordan VC, Murphy CS. Endocrine pharmacology of antiestrogens as antitumor agents. *Endocr Rev* 1990;11:578–610.
- Musgrove EA, Hamilton JA, Lee CS, Sweeney KJ, Watts CK, Sutherland RL. Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. *Mol Cell Biol* 1993;13:3577–87.
- Horwitz KB, McGuire WL, Pearson OH, Segaloff A. Predicting response to endocrine therapy in human breast cancer: a hypothesis. *Science* 1975; 189:726–7.
- Pritchard KI, Sutherland DJ. The use of endocrine therapy. *Hematol Oncol Clin North Am* 1989;3:765–805.
- Santen RJ, Manni A, Harvey H, Redmond C. Endocrine treatment of breast cancer in women. *Endocr Rev* 1990;11:221–65.
- Early Breast Cancer Trialists' Collaborative Group. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 Randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. *Lancet* 1992;339:1–15; 71–85.
- Early Breast Cancer Trialists' Collaborative Group. Tamoxifen for early breast cancer: an overview of the randomised trials. *Lancet* 1998;351: 1451–67.
- Jaiyesimi IA, Buzdar AU, Decker DA, Hortobagyi GN. Use of tamoxifen for breast cancer: twenty-eight years later. *J Clin Oncol* 1995;13:513–29.
- Foekens JA, Schmitt M, Van Putten WL, Peters HA, Kramer MD, Janicke F, et al. Plasminogen activator inhibitor-1 and prognosis in primary breast cancer. *J Clin Oncol* 1994;12:1648–58.
- Osborne CK, Coronado E, Allred DC, Wiebe V, DeGregorio M. Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *J Natl Cancer Inst* 1991;83:1477–82.
- Johnston SR, Haynes BP, Smith IE, Jarman M, Sacks NP, Ebbs SR, et al. Acquired tamoxifen resistance in human breast cancer and reduced intratumoral drug concentration. *Lancet* 1993;342:1521–2.
- Daffada AA, Johnston SR, Smith IE, Detre S, King N, Dowsett M. Exon 5 deletion variant ER messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/ps2 status in human breast cancer. *Cancer Res* 1995;55:288–93.
- Fuqua SA, Wolf DM. Molecular aspects of estrogen receptor variants in breast cancer. *Breast Cancer Res Treat* 1995;35:233–41.
- Murphy LC, Hilsenbeck SG, Dotzlaw H, Fuqua SA. Relationship of clone 4 estrogen receptor variant messenger RNA expression to some known prognostic variables in human breast cancer. *Clin Cancer Res* 1995;1: 155–9.
- Clarke R, Dickson RB, Lippman ME. Hormonal aspects of breast cancer.

- Growth factors, drugs and stromal interactions. *Crit Rev Oncol Hematol* 1992;12:1–23.
- (34) Dorssers LC, Van Aghoven T, Sieuwerts AM. Genetic mechanisms involved in progression to hormone independence of human breast cancer. In: Berns PM, Romijn JC, Schroeder FH, editors. *Mechanisms of progression to hormone-independent growth of breast and prostatic cancer*. Carnforth (U.K.): Parthenon Publishing Group Ltd; 1991. p. 169–82.
- (35) Clarke R, Thompson EW, Leonessa F, Lippman J, McGarvey M, Frandsen TL, et al. Hormone resistance, invasiveness, and metastatic potential in breast cancer. *Breast Cancer Res Treat* 1993;24:227–39.
- (36) Dorssers LC, Van Aghoven T, Dekker A, Van Aghoven TL, Kok EM. Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. *Mol Endocrinol* 1993;7:870–8.
- (37) Dorssers LC, Veldscholte J. Identification of a novel breast-cancer-antiestrogen-resistance (BCAR2) locus by cell-fusion-mediated gene transfer in human breast-cancer cells. *Int J Cancer* 1997;72:700–5.
- (38) Van Aghoven T, Van Aghoven TL, Portengen H, Foekens JA, Dorssers LC. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. *Cancer Res* 1992;52:5082–8.
- (39) Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. *BioTechniques* 1989;7:980–90.
- (40) Van den Ouweland AM, Breuer ML, Steenbergh PH, Schalken JA, Bloemers HP, Van de Ven WJ. Comparative analysis of the human and feline c-sis proto-oncogenes: identification of 5' human c-sis coding sequences that are not homologous to the transforming gene of simian sarcoma virus. *Biochim Biophys Acta* 1985;825:140–7.
- (41) Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. New York (NY): Cold Spring Harbor Press; 1989.
- (42) Van Aghoven T, Van Aghoven TL, Dekker A, Van der Spek PJ, Vreede L, Dorssers LC. Identification of BCAR3 by a random search for genes involved in antiestrogen resistance of human breast cancer cells. *EMBO J* 1998;17:2799–808.
- (43) Van Aghoven T, Van Aghoven TL, Dekker A, Foekens JA, Dorssers LC. Induction of estrogen independence of ZR-75-1 human breast cancer cells by epigenetic alterations. *Mol Endocrinol* 1994;8:1474–83.
- (44) Sander EE, van Delft S, ten Klooster JP, Reid T, van der Kammen RA, Michiels F, et al. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell–cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J Cell Biol* 1998;143:1385–98.
- (45) van der Flier S, Brinkman A, Look MP, Kok EM, Meijer-van Gelder ME, Klijn GM, et al. Bcar1/p130Cas protein and primary breast cancer: prognosis and response to tamoxifen treatment. *J Natl Cancer Inst* 2000;92:000–000.
- (46) Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, et al. A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO J* 1994;13:3748–56.
- (47) Kozak M. An analysis of vertebrate mRNA sequences: intimations of translational control. *J Cell Biol* 1991;115:887–903.
- (48) Nakamoto T, Sakai R, Ozawa K, Yazaki Y, Hirai H. Direct binding of C-terminal region of p130^{Cas} to SH2 and SH3 domains of Src kinase. *J Biol Chem* 1996;271:8959–65.
- (49) Polte TR, Hanks SK. Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130^{Cas}. *Proc Natl Acad Sci U S A* 1995;92:10678–82.
- (50) Callen DF, Lane SA, Kozman H, Kremmidiotis G, Whitmore SA, Lowenstein M, et al. Integration of transcript and genetic maps of chromosome 16 at near-1-Mb resolution: demonstration of a “hot spot” for recombination at 16p12. *Genomics* 1995;29:503–11.
- (51) Schlaepfer DD, Broome MA, Hunter T. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130^{Cas}, and Nck adaptor proteins. *Mol Cell Biol* 1997;17:1702–13.
- (52) Liu F, Sells MA, Chernoff J. Transformation suppression by protein tyrosine phosphatase 1B requires a functional SH3 ligand. *Mol Cell Biol* 1998;18:250–9.
- (53) Blaukat A, Ivankovic-Dikic I, Gronroos E, Dolfi F, Tokiwa G, Vuori K, et al. Adaptor proteins grb2 and crk couple pyk2 with activation of specific mitogen-activated protein kinase cascades. *J Biol Chem* 1999;274:14893–901.
- (54) Lu Y, Brush J, Stewart TA. NSP1 defines a novel family of adaptor proteins linking integrin and tyrosine kinase receptors to the c-Jun N-terminal kinase/stress-activated protein kinase signaling pathway. *J Biol Chem* 1999;274:10047–52.
- (55) Jonkers J, Berns A. Retroviral insertional mutagenesis as a strategy to identify cancer genes. *Biochim Biophys Acta Rev Cancer* 1996;1287:29–57.
- (56) Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Nishida J, et al. Characterization, partial purification, and peptide sequencing of p130, the main phosphoprotein associated with v-Crk oncoprotein. *J Biol Chem* 1994;269:32740–6.
- (57) Auvinen M, Paasinen-Sohns A, Hirai H, Andersson LC, Holtta E. Ornithine decarboxylase- and ras-induced cell transformations: reversal by protein tyrosine kinase inhibitors and role of pp130^{CAS}. *Mol Cell Biol* 1995;15:6513–25.
- (58) Honda H, Oda H, Nakamoto T, Honda Z, Sakai R, Suzuki T, et al. Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. *Nat Genet* 1998;19:361–5.
- (59) Harte MT, Hildebrand JD, Burnham MR, Bouton AH, Parsons JT. p130^{Cas}, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *J Biol Chem* 1996;271:13649–55.
- (60) Nakamoto T, Sakai R, Honda H, Ogawa S, Ueno H, Suzuki T, et al. Requirements for localization of p130^{CAS} to focal adhesions. *Mol Cell Biol* 1997;17:3884–97.
- (61) Nojima Y, Morino N, Mimura T, Hamasaki K, Furuya H, Sakai R, et al. Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130^{Cas}, a Src homology 3-containing molecule having multiple Src homology 2-binding motifs. *J Biol Chem* 1995;270:15398–402.
- (62) Vuori K, Hirai H, Aizawa S, Ruoslahti E. Induction of p130^{Cas} signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Mol Cell Biol* 1996;16:2606–13.
- (63) Howe A, Aplin AE, Alahari SK, Juliano RL. Integrin signaling and cell growth control. *Curr Opin Cell Biol* 1998;10:220–31.
- (64) Schlaepfer DD, Hunter T. Integrin signalling and tyrosine phosphorylation: just the FAKs? *Trends Cell Biol* 1998;8:151–7.
- (65) Ojaniemi M, Vuori K. Epidermal growth factor modulates tyrosine phosphorylation of p130^{Cas}. Involvement of phosphatidylinositol 3'-kinase and actin cytoskeleton. *J Biol Chem* 1997;272:25993–8.
- (66) Rozengurt EV. Gastrointestinal peptide signaling through tyrosine phosphorylation of focal adhesion proteins. *Am J Physiol* 1998;275:G177–82.
- (67) Sorokin A, Reed E. Insulin stimulates the tyrosine dephosphorylation of docking protein p130cas (Crk-associated substrate), promoting the switch of the adaptor protein crk from p130cas to newly phosphorylated insulin receptor substrate-1. *Biochem J* 1998;334:595–600.
- (68) Murakami H, Iwashita T, Asai N, Iwata Y, Narumiya S, Takahashi M. Rho-dependent and -independent tyrosine phosphorylation of focal adhesion kinase, paxillin and p130Cas mediated by Ret kinase. *Oncogene* 1999;18:1975–82.
- (69) Manie SN, Beck AR, Astier A, Law SF, Cauty T, Hirai H, et al. Involvement of p130^{Cas} and p105^{HEF1}, a novel Cas-like docking protein, in a cytoskeleton-dependent signaling pathway initiated by ligation of integrin or antigen receptor on human B cells. *J Biol Chem* 1997;272:4230–6.
- (70) Cary LA, Han DC, Polte TR, Hanks SK, Guan JL. Identification of p130^{Cas} as a mediator of focal adhesion kinase-promoted cell migration. *J Cell Biol* 1998;140:211–21.
- (71) Klemke RL, Leng J, Molander R, Brooks PC, Vuori K, Cheresch DA. CAS/Crk coupling serves as a “molecular switch” for induction of cell migration. *J Cell Biol* 1998;140:961–72.
- (72) Tamura M, Gu J, Takino T, Yamada KM. Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. *Cancer Res* 1999;59:442–9.
- (73) Black DS, Bliska JB. Identification of p130^{Cas} as a substrate of *Yersinia* YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. *EMBO J* 1997;16:2730–44.
- (74) Persson C, Carballeira N, Wolf-Watz H, Fallman M. The PTPase YopH inhibits uptake of *Yersinia*, tyrosine phosphorylation of p130^{Cas} and FAK, and the associated accumulation of these proteins in peripheral focal adhesions. *EMBO J* 1997;16:2307–18.

- (75) Pawson T, Scott JD. Signaling through scaffold, anchoring, and adaptor proteins. *Science* 1997;278:2075–80.
- (76) Law SF, Estojak J, Wang BL, Mysliwiec T, Kruh G, Golemis EA. Human enhancer of filamentation 1, a novel p130^{cas}-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1996;16:3327–37.
- (77) Minegishi M, Tachibana K, Sato T, Iwata S, Nojima Y, Morimoto C. Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in β 1 integrin-mediated signaling in lymphocytes. *J Exp Med* 1996;184:1365–75.
- (78) Ishino M, Ohba T, Sasaki H, Sasaki T. Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn. *Oncogene* 1995;11:2331–8.
- (79) Alexandropoulos K, Baltimore D. Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel, p130^{Cas}-related protein. *Sin. Genes Dev* 1996;10:1341–55.
- (80) Law SF, Zhang YZ, Klein-Szanto AJ, Golemis EA. Cell cycle-regulated processing of HEF1 to multiple protein forms differentially targeted to multiple subcellular compartments. *Mol Cell Biol* 1998;18:3540–51.
- (81) Astier A, Avraham H, Manie SN, Groopman J, Canty T, Avraham S, et al. The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after β 1-integrin stimulation in B cells and binds to p130^{Cas}. *J Biol Chem* 1997;272:228–32.
- (82) Garton AJ, Burnham MR, Bouton AH, Tonks NK. Association of PTP-PEST with the SH3 domain of p130^{Cas}; a novel mechanism of protein tyrosine phosphatase substrate recognition. *Oncogene* 1997;15:877–85.
- (83) Ohba T, Ishino M, Aoto H, Sasaki T. Interaction of two proline-rich sequences of cell adhesion kinase beta with SH3 domains of p130Cas-related proteins and a GTPase-activating protein. *Graf. Biochem J* 1998;330:1249–54.
- (84) Burnham MR, Harte MT, Richardson A, Parsons JT, Bouton AH. The identification of p130^{Cas}-binding proteins and their role in cellular transformation. *Oncogene* 1996;12:2467–72.
- (85) Khwaja A, Hallberg B, Warne PH, Downward J. Networks of interaction of p120^{cb1} and p130^{Cas} with Crk and Grb2 adaptor proteins. *Oncogene* 1996;12:2491–8.

NOTES

Supported by grant DDHK 94-847 from the Dutch Cancer Foundation.

We thank Drs. M. Look, J. Veldscholte, and M. Schutte for critically reviewing this manuscript. We also thank Drs. J. A. Foekens and T. van Agthoven for their stimulating discussions, Dr. K. Nooter and K. van Wingerden for their contribution to the testing of drugs, Dr. J. G. Collard for the gift of the episomal expression vector, and Dr. A. M. Cleton-Jansen for her assistance with the analysis of the somatic cell hybrid panel.

Manuscript received June 9, 1999; revised November 3, 1999; accepted November 9, 1999.