Identification of *BCAR3* by a random search for genes involved in antiestrogen resistance of human breast cancer cells

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The antiestrogen tamoxifen is important in the treatment of hormone-dependent breast cancer, although development of resistance is inevitable. To unravel the molecular mechanisms of antiestrogen resistance, a search for involved genes was initiated. Retrovirus-mediated insertional mutagenesis was applied to human ZR-75-1 breast cancer cells. Infected cells were subjected to tamoxifen selection and a panel of resistant cell clones was established. Screening for a common integration site resulted in the identification of a novel gene designated *BCAR3*. Transfer of this locus by cell fusion or transfection of the *BCAR3* cDNA to ZR-75-1 and MCF-7 cells induces antiestrogen resistance. *BCAR3* represents a putative SH2 domain-containing protein and is partly homologous to the cell division cycle protein CDC48.

Keywords: breast cancer/CDC48/estrogen receptor/SH2 domain/tamoxifen

Introduction

Growth and differentiation of the mammary gland is regulated by complex interactions between hormones and polypeptide growth factors with their specific receptors (Osborne, 1987; Mauvais-Jarvis *et al.*, 1988; Clarke *et al.*, 1992). Disruption of these strictly controlled signaling pathways may result in the development of cancer. It is widely accepted that breast tumors initially are dependent upon estrogens for establishment and progression (Jensen, 1981; Horwitz, 1993). Treatment of hormone-dependent breast cancer with antiestrogens like tamoxifen can delay recurrence or induce remissions of the disease (Early Breast Cancer Trial Collab Group, 1992). Antiestrogens compete with estrogen for the estrogen receptor (ER) (Jordan and Murphy, 1990; Jordan, 1994; Wolf and Fuqua, 1996), resulting in blocking of hormone signaling and growth inhibition of the tumor cells (Jordan and Murphy, 1990; Musgrove *et al.*, 1993). Unfortunately, nearly all patients who initially respond to antiestrogen therapy

experience a relapse due to the development of antiestrogen-resistant metastases. Understanding the mechanisms of drug resistance may lead to the development of new treatment strategies. A number of possibilities for tamoxifen resistance have been postulated (reviewed by King, 1990; Johnston *et al.*, 1992; Horwitz, 1993; Wolf and Jordan, 1993; Clarke *et al.*, 1994; Jordan, 1994; Klijn *et al.*, 1994; Osborne and Fuqua, 1994; Clarke and Brnner, 1995), including loss of ER, alterations in structure and/or function of the ER, tamoxifen-stimulated growth, altered drug metabolism and overexpression of different genes. However, the general mechanisms underlying the resistance for the majority of breast cancers are still not known. Progression of breast cancer to hormone insensitivity may be the result of a mutational event, followed by clonal selection (Dorssers *et al.*, 1991; Clarke *et al.*, 1993). Alternatively, the altered phenotype may be the result of epigenetic mechanisms involving stable changes in a large number of tumor cells (Van Agthoven *et al.*, 1994).

A random search for genes involved in antiestrogen resistance was initiated. Due to the inability to cultivate primary breast tumor cells in large quantities for prolonged times, the human breast cancer cell line ZR-75-1 (Engel *et al.*, 1978) was selected as a model for hormone-dependent breast cancer. This cell line is strictly estrogen dependent for proliferation and can be inhibited fully by antiestrogens (Van Agthoven *et al.*, 1992; Dorssers *et al.*, 1993). Previously, we have shown that up-regulation of expression of a single gene is sufficient to induce estrogen independence. Transduction of estrogen-dependent ZR-75-1 cells with an expression vector carrying the epidermal growth factor (EGF) receptor resulted in proliferation independent of estradiol (Van Agthoven *et al.*, 1992). Furthermore, in different experiments, we showed that random changes in the methylation pattern of the DNA, induced by 5-azacytidine, resulted in a high frequency of antiestrogen-resistant cell clones (Van Agthoven *et al.*, 1994). These results indicate that several escape mechanisms of hormone dependence are possible. However, these strategies do not readily allow for the isolation of the involved genes. Hence, identification of (novel) genes connected with the development of antiestrogen resistance was approached by retrovirus-mediated insertional mutagenesis (Dorssers *et al.*, 1993). Random integration of retroviruses may lead to alteration of expression or truncation of genes in the vicinity of the integration site. Consequently, insertional mutagenesis can mimic changes leading to tumor progression. The provirus, carrying long terminal repeats (LTRs) and a neomycin resistance (NeoR) gene, serves as a unique tag to identify genes in the proximity of the integration site. This approach has been successful in the identification of transforming genes in the mouse (Habets *et al.*, 1994; Jonkers and Berns, 1996), and preliminary results show that this strategy may be

useful for the identification for drug resistance genes involved in human disease (Dorssers *et al.*, 1993; Dorssers and Veldscholte, 1997). ZR-75-1 cells (8×10^8) were infected with defective retroviruses and subsequently selected for growth in the presence of 4-hydroxy-tamoxifen, the active metabolite of tamoxifen. Eighty 4-hydroxy-tamoxifen-resistant cell clones have been isolated and established as stable cell lines. Many of the resistant clones carry more than one integrated provirus. The presence of a retroviral genome integrated in a particular locus of several cell clones which have arisen independently (common integration site) strongly suggests that a specific gene function is altered by the viral integration and contributes to the development of antiestrogen resistance. Therefore, a search for common sites of integration in the established panel of resistant cell clones was performed to identify the loci of interest. This has resulted in the identification of three chromosomal loci, designated breast cancer antiestrogen resistance (BCAR) locus 1 (Dorssers *et al.*, 1993), 2 (Dorssers and Veldscholte, 1997) and 3. This report describes the cloning and characterization of the BCAR3 gene, involved in antiestrogen resistance, identified by retrovirus-mediated insertional mutagenesis.

Results

Common site of integration BCAR3

To identify genes involved in antiestrogen resistance, cell clone X-3-6 was selected from our panel of 80 cell clones resistant to 4-hydroxy-tamoxifen. Cell clone X-3-6 contains only one integrated provirus which is expected to induce the resistant phenotype. Furthermore, cell clone X-3-6 does not belong to the clones which contain a viral integration in the BCAR1 or BCAR2 loci. To screen for additional clones with an insertion in this region, an integration site-specific probe was created by inverse PCR (Figure 1A). Chromosomal DNA of clone X-3-6, digested with a panel of restriction enzymes, was analyzed by Southern blotting techniques. Hybridization with a NeoR probe, specific for the retroviral integration, showed that a combination of *Bam*HI and *Bgl*II digestion resulted in a restriction fragment of ~6.5 kb, convenient for isolation of a large fragment adjacent to the viral integration (Figure 1A). DNA of cell clone X-3-6 cleaved with *Bam*HI and *Bgl*II restriction enzymes was circularized by ligation and amplified using outward-directed primers located within the NeoR and viral *gag* sequences. Subsequently, this inverse PCR product was amplified with two sets of nested primers located within the LTRs (Figure 1A). This approach resulted in a 3.2 kb PCR fragment of genomic sequences adjacent to the viral integration site. Alu-repetitive sequences were removed from this fragment by subcloning, which resulted in a 1.6 kb probe (Figure 1A). Southern analysis of X-3-6 and parental ZR-75-1 DNA digested with a panel of restriction enzymes and hybridized with the integration probe and a NeoR probe confirmed the specificity of this probe for the X-3-6 integration site. Subsequently, the cell line panel was screened for the occurrence of a common site of integration. Southern blots of the 80 antiestrogen-resistant cell lines containing DNA, digested with either *Bam*HI, *Bgl*II or *Hind*III, were hybridized with the integration-specific probe of clone

Fig. 1. (A) Genomic analysis of the BCAR3 integration locus. The primers used for inverse PCR to obtain an integration site-specific probe are indicated below the LN virus (open arrows). The position and orientation of the different retroviral integrations are indicated with an arrow. Mapping of the different integrations was performed by Southern blotting techniques and PCR analysis with primers located on the 3' LTR and on the integration probe (open arrows). The positions of the integrations are mapped with respect to the retroviral integration in cell clone X-3-6. The integration site-specific probe is marked. The exon detected near the integrations is denoted as a black box. Restriction sites *Bcl*I and *Bgl*II (B) and *Hind*III (H) and the *gag* region are indicated. (B) Common integration site. A Southern blot with genomic DNA of cell lines digested with the restriction enzyme *Hind*III was hybridized with the BCAR3 integration probe. Roman numerals indicate the different BCAR3 cell clones. In the six independently derived BCAR3 cell lines of a total panel of 80 cell lines, an additional larger restriction fragment is detected as a result of the integration of the provirus. The intensity of the additional band in cell clone XIV-2-4C is less in comparison with the other cell lines. This may be explained by contamination with a different clone during isolation. Clone XIV-2-4C was omitted from further (RNA) analysis.

X-3-6. Figure 1B shows that this probe identified a 15 kb *Hind*III restriction fragment in the parental ZR-75-1 cells and in the derived integration cell clones. In the X-3-6 cell clone, an additional fragment (~18 kb) was observed, resulting from integration of the LN retrovirus (~3 kb) in the other allele (Figure 1B). This analysis revealed five additional cell clones with a provirus integrated in this region. In total, a region of ~25 kb surrounding the X-3-6 integration site was analyzed. Additional integrations further up- or downstream of this region cannot be excluded. Detailed mapping analysis of the six integration sites demonstrated that they were distinct and thus confirmed the independent origin of the clones (Figure 1A). In addition, Southern analysis and PCR mapping showed that all clones have a similar 5' to 3' direction of transcription of the integrated retrovirus. Five viral integrations have occurred in a 1 kb region and one, in clone X-1-2B, is integrated 5 kb upstream. The occurrence of six retroviral insertions in six cell lines which have arisen independently in a panel of 80 cell lines in a region of 5 kb is unlikely to be the result of a random process (2.9×10^{-19} , binomial distribution) (Dorssers *et al.*, 1993). This common site of integration is thus tightly linked with antiestrogen resistance *in vitro* and is termed the breast cancer antiestrogen resistance 3 (BCAR3) locus.

Fig. 2. Transfer of the *BCAR3* locus. Somatic cell hybrids with (A) or without (B) the *BCAR3* locus were cultured in the presence of 4-hydroxy tamoxifen. Representative examples were stained with hematoxylin–eosin at day 10. Hybrid cells with the *BCAR3* locus (A) proliferate in medium containing 4-hydroxy-tamoxifen, while hybrid cells with the non-relevant integration locus (B) are fully inhibited by 4-hydroxy-tamoxifen.

Transfer of the *BCAR3* locus by somatic cell fusion

Subsequently, we wished to determine whether the alteration in the *BCAR3* locus, caused by retroviral mutagenesis, is a dominant or recessive event. Southern blotting analysis showed that one of the integration clones, XI-1-6A, carries two integrated proviruses, one in the *BCAR3* locus and an additional integration which is expected to be non-relevant for antiestrogen resistance. This cell line was chosen for generation of somatic cell hybrids between a hygromycin B-resistant subclone of the parental ZR-75-1 cells (ZH3D7) and XI-1-6A cells. The presence of the additional proviral integration in this cell line serves as an internal control. An approach was chosen in which the donor cells were lethally irradiated. Somatic cell hybrids were selected with hygromycin B and G418, resulting solely in hybrids consisting of parental ZR-75-1 cells which have acquired at least one of the NeoR-containing integration loci. In total, 11 hybrids were obtained which were characterized with an informative *HindIII* restriction digestion to distinguish between the two viral integrations. This analysis showed that five hybrids contained the locus with the viral integration in the *BCAR3* locus and six hybrids carried the other viral integration site. Subsequently, we tested whether the hybrids were antiestrogen resistant. The growth performance of triplicate cultures of the cell hybrids was compared with the parental ZR-75-1, ZH3D7 and XI-1-6A cells in culture medium containing 1 μ M of 4-hydroxy-tamoxifen. Antiestrogen sensitivity of cell hybrids was scored on days 7 and 10. Cultures of hybrid cells showing poor, rounded cell morphology, loose

attachment to the culture flask, low numbers of mitoses and little or no increase in cell numbers (measured as the percentage of covered surface, ~20–30%) comparable with ZR-75-1 and ZH3D7 cells were scored 4-hydroxy-tamoxifen sensitive (Figure 2B). Hybrids which looked healthy, with a flattened morphology, firmly attached to the culture flask with readily visible numbers of mitoses, and cell numbers occupying up to 90% of surface of the culture flask, comparable with XI-1-6A cells, were scored tamoxifen resistant (Figure 2A). This analysis showed that only the five hybrid cell lines carrying the *BCAR3* locus were resistant to 4-hydroxy-tamoxifen. These results indicate that transfer of the *BCAR3* integration locus instantly confers dominant resistance to the parental ZR-75-1 cells.

Identification of coding sequences in the *BCAR3* locus

In five out of six resistant cell lines, the integration of the retrovirus occurred in a very narrow region (Figure 1). This indicates a positive selection for this region and a common mechanism of alteration of gene expression. Northern blots with RNA of the integration clones were screened with the genomic inverse PCR probe to identify possible transcribed sequences. Large transcripts were observed in the *BCAR3* clones, which vary in size in the various cell clones and were not detected in the parental ZR-75-1 cells (Figure 3). Northern blot hybridization demonstrated that these are chimeric transcripts composed of LTR, NeoR and *BCAR3* sequences (data not shown). The observed differences in mRNA sizes in individual

Fig. 3. mRNA expression of *BCAR3*. Northern blots containing total RNA from ZR-75-1 cells, antiestrogen-resistant cell clones and MDA-MB-231 breast cancer cells were hybridized with a *BCAR3* probe. ZR-75-1 cells were cultured in the presence (E2) or absence (BCS) of 1 nM 17 β -estradiol or in medium containing 1 μ M 4-hydroxy-tamoxifen (TAM). Antiestrogen-resistant cell clones were cultured in the presence of 4-hydroxy-tamoxifen. The positions of 18S and 28S rRNAs are indicated.

cell clones can be explained by the position of the integrated retroviruses relative to the *BCAR3* sequences and readthrough transcripts from the viral LTR (Figures 1 and 3). More importantly, screening of Northern blots with RNA of a panel of cell lines revealed a transcript of ~3.4 kb in the estrogen-independent MDA-MB-231 (Cailleau *et al.*, 1974) breast cancer cell line (Figure 3). This indicates that this genomic integration probe contains exon sequences. This *BCAR3* transcript appears also to be present in the integration clones and is barely detectable in the ZR-75-1 cells. Estrogens can modulate gene expression of target genes. However, *BCAR3* is not regulated detectably in ZR-75-1 cells. Cultivation of ZR-75-1 cells in the presence and absence of estradiol and in the presence of 4-hydroxy-tamoxifen did not result in up-regulation of *BCAR3* expression (Figure 3). Therefore, it is most likely that the observed up-regulation of the *BCAR3* RNA is induced by the integrated virus.

***BCAR3* cDNA cloning and predicted amino acid sequence**

To isolate the gene corresponding to the observed transcript, a human testis cDNA library was screened with the integration site-specific probe. In the first screening round, a 1.9 kb cDNA clone was isolated (#16, Figure 4A). Sequence comparison of the genomic integration probe with the cDNA showed that the genomic probe contains a 133 bp exon, identical to sequences of the cDNA clone #16 (represented by nucleotides 456–589 in Figure 4B) with consensus splice donor and acceptor sites. Northern blot analysis confirmed that this cDNA clone recognizes the *BCAR3* transcripts. In successive screening efforts to obtain the full-length cDNA, overlapping clones were isolated (Figure 4A). The assembled cDNA clones consist of 3004 bp, while the mRNA is ~3.4 kb as estimated on Northern blots. The first ATG is not preceded by an in-frame stop codon. Therefore, a 5' RACE (rapid amplification of cDNA ends) strategy was performed to obtain the additional sequences at the 5' end of the *BCAR3* mRNA.

Total RNA of MDA-MB-231 cells, which show abundant expression of *BCAR3*, was used to obtain the full coding region of *BCAR3*. Several 5' RACE clones were isolated and sequenced. It was shown that an in-frame stop codon (TGA) is present 72 bp upstream of the first ATG. The cDNA obtained is 3042 bp in total and contains the complete coding region. Three ATGs are present in the first 174 bp of the cDNA. Analysis of the surrounding sequences of the three putative start sites indicates that the first ATG codon at position 99 fits the Kozak consensus sequence (Kozak, 1991). The open reading frame (ORF) is flanked by an in-frame TGA translation termination codon at position 2574, followed by a 448 nucleotide 3'-untranslated region and a poly(A) tail of 19 nucleotides. *BCAR3* has a single ORF that encodes a protein of 825 amino acids with a predicted molecular mass of 92 kDa, assuming that translation starts at the first ATG (Figure 4B). Comparison of the sequence of *BCAR3* with the available protein and nucleotide databases revealed that *BCAR3* is a newly identified gene whose product has a single stretch of amino acids (codons 154–253 in Figure 4) with strong homology with Src homology 2 (SH2) domains of other proteins (Figure 4C). A profile scan utilizing the Prosite profile database detected no further putative protein domains. However, sequences homologous to part of the yeast cell division cycle protein CDC48 were observed by using the BLASTP algorithm (codons 699–812, Figure 4D).

mRNA expression of *BCAR3*

We next evaluated the expression of *BCAR3* mRNA in various normal tissues. For this purpose, commercially available Northern blots were hybridized with the 1.9 kb cDNA probe clone #16. *BCAR3* mRNA is widely expressed, and abundant transcripts were observed in heart, placenta, skeletal muscle, spleen, prostate, testis, ovary, small intestine, colon and fetal kidney. In skeletal muscle and heart, an additional 6 kb mRNA is present (Figure 5). The nature of this larger transcript is at present unclear but may be explained by alternative splicing. In addition, Northern analysis was performed on RNA isolated from non-malignant breast tissues. No expression of *BCAR3* was detected in these samples ($n = 6$). However, the amount of luminal epithelial cells in normal breast is small compared with stromal cells. Therefore, it cannot be excluded that *BCAR3* is expressed by the glandular epithelial cells. A panel of cell lines originating from breast, ovarian and endometrial cancer was screened by Northern analysis for expression of *BCAR3*. These results were compared with ER and EGF receptor protein expression (Table I). In the cell lines, only the 3.4 kb *BCAR3* mRNA was detected. In the breast cancer cell lines tested (ZR-75-1, BT-474, MCF-7, T47D, ER-positive; EVSA-T, SK-BR-3, MDA-MB-134, ER-negative), no expression of *BCAR3* was detected. In the ER-negative breast cancer cells BT-20, HS-578 and MDA-MB-231, abundant expression of *BCAR3* was observed. In two ER-negative immortalized mammary epithelial cell lines, RC-6 and HBL-100, only the latter showed expression. In ovarian cell lines (SKOV3, SKOV6, OVCAR3, 2774, 2780, HOC7 and KB3.1, all ER-negative), expression of *BCAR3* was observed except for in 2780. In the endometrial cell lines tested (SCRC, AN-3-CA, HEC1A, HEC1B, RL 95-2 and

Fig. 4. Structure of *BCAR3*. (A) Schematic representation of the *BCAR3* gene. The thick lines represent the overlapping cDNAs. The arrow indicates the position of the viral integration sites in the genome compared with the cDNA sequence. The exon detected near the integrations is indicated (black box, see also Figure 1A). The hatched box indicates the 5' region obtained by RACE. The location of the putative SH2 domain and the region homologous to CDC48 is shown. (B) *BCAR3* deduced amino acid sequence. The amino acid sequence is given in the one-letter code. The predicted N-terminal methionine and the ATG codon (M) of the shortened product observed in the *BCAR3* cell clones and the first stop codon (*) are indicated. The region of similarity to other SH2 domain-containing proteins is underlined and the region homologous to CDC48 is double underlined. (C) Sequence alignment of the SH2 domain of the human *BCAR3* and other human SH2-containing proteins. The conserved residues of the *BCAR3*, *Shc*, *Grb2*, *Fer*, *Fes*, *Hck* and *Src* proteins are compared by giving priority to *BCAR3*. Sequences used in this figure are *Shc*, SWISS-PROT database accession No. P29353, *Grb2* P29354, *Fer* P16591, *Fes* P07332, *Hck* P08631 and *Src* G338460. Identical amino acids are represented by black boxes, whereas similar residues (A, G, S, P, T; D, E, N, Q; H, K, R; I, L, M, V; F, Y, W) are given in gray boxes. Key residues in specific interaction with phosphotyrosine (*) and substrate specificity-determining residues (#) in *Src* are indicated (Songyang *et al.*, 1994; Cohen *et al.*, 1995). (D) Sequence alignment of the CDC48-related domain of the human *BCAR3* protein. The CDC48-related residues of the *BCAR3* protein are visualized by giving priority to *BCAR3*. The CDC48 equivalents are derived from the following species *Saccharomyces cerevisiae* (Sc) accession No. A39977, *Caenorhabditis elegans* (Ce) P54811, *Xenopus laevis* (Xl) P23787, *Mus musculus* (Mm) Q01853, *Arabidopsis thaliana* (At) P54609, *Sulfolobus acidocaldarius* (Sa) Q07590, *Homo sapiens* (Hs) ESTs AA009995 and N72512.

Fig. 5. The expression of *BCAR3* mRNA in various normal human tissues. Northern blots (Clontech) containing 2 µg of poly(A)⁺ RNA from the indicated tissues were hybridized with *BCAR3* cDNA probe, clone #16. The mobility of RNA molecular size standards is indicated on the left in kilobases.

Table 1. Expression of *BCAR3* mRNA was compared with expression of ER and EGF receptor protein

Cell line	BCAR3	ER	EGFR
Breast			
ZR-75-1	–	+	–
MCF-7	–	+	–
T-47D	–	+	+
BT-474	–	+	nd
BT-20	++	–	nd
EVSA-T	–	–	+
HS-578-T	+	–	nd
MDA-MB-134	–	–	nd
MDA-MB-231	+++	–	+
SK-BR-3	–	–	+
HBL-100	+	–	nd
RC-6	–	–	nd
Ovary			
2780	–	–	+
2774	++	–	+
HOC-7	+	–	+
KB3.1	+	–	nd
OVCAR-3	+	–	+
SK-OV-3	+	–	+
SK-OV-6	+	–	+
Endometrium			
ECC-1	–	+	+
AN-3-CA	+	–	+
HEC-1-A	++	–	+
HEC-1-B	+	–	+
KLE	–	–	+
SCRC	++	–	+
RL 95-2	++	–	+

Receptor concentrations determined with biochemical assays are expressed as fmol/mg protein. Expression of ER >10 fmol/mg and EGF receptor >50 fmol/ml of protein was scored positive (+). ND not done. (*) Expression determined by Northern analysis. Expression levels of *BCAR3* mRNA varied between –, no expression detected after 7 days exposure and +++, high expression detected after overnight exposure on film.

KLE, ER-negative and ECC1, ER-positive), high mRNA levels were observed except for in ECC1 and KLE. The integrity of the mRNA on Northern blots was examined by hybridization to a GAPDH or actin probe. Correlation of ER and EGF receptor expression with expression of

Fig. 6. *In vitro* transcription translation. *In vitro* translated full-length *BCAR3* (B3) was compared with the shortened form (B3s) of *BCAR3*, as deduced from analyses of the virus clones (nucleotides 383–2951). Control reactions with a luciferase construct (61 kDa product, 10% loaded) and reaction without plasmid DNA (–) are shown. Detection was by chemiluminescence. The mobility of molecular size standards is indicated on the left in kilodaltons.

BCAR3 showed an inverse relationship between expression of *BCAR3* and ER (McNemar test, $P = 0.025$).

In vitro transcription translation of *BCAR3*

Figures 1 and 4 show that all proviral integrations had occurred in an intron in the 5' region of the *BCAR3* gene, most likely resulting in a shorter mRNA. Northern blot analysis with a specific probe for the 5' part of the gene (represented by nucleotides 38–420) demonstrated that these sequences were not present in the shortened *BCAR3* mRNA of the integration clones, but were present in the MDA-MB-231 *BCAR3* transcripts (not shown). The shortened *BCAR3* gene in the antiestrogen-resistant clones may encode a protein of 699 amino acids with a predicted mass of 78 kDa, assuming that the ATG codon at position 439 is the initiator methionine. Both *BCAR3* and a shortened construct (represented by nucleotides 421–2989) resembling the presumed situation in the *BCAR3* clones were subcloned in a vector which allows the expressed protein to start with the authentic ATG codon. *In vitro* transcription translated *BCAR3* migrated as a single band by SDS-PAGE at ~90 kDa (Figure 6). *In vitro* transcription translation analysis of the shortened construct showed a single band of ~74 kDa (Figure 6).

Ectopic expression of BCAR3 induces estrogen independence in ZR-75-1 and MCF-7 cells

Stable transfectants with the *BCAR3* gene were established to provide conclusive evidence that *BCAR3* is the gene responsible for antiestrogen resistance in this locus. For this purpose, two expression constructs were made in which the *BCAR3* cDNA #32, containing the complete coding region, was positioned under control of a cytomegalovirus (CMV) and a LTR promoter. These expression vectors were transfected into ZR-75-1 cells and stable G418-resistant clones were generated and isolated. These transfectants, designated ZR/BCAR3 cells, were expanded in the presence of 17β-estradiol to certify estrogen dependence. We next determined the proliferation capacity of the *BCAR3* transfectants in the presence of the antiestrogen 4-hydroxy-tamoxifen. In Figure 7, the increase in cell numbers over an 11 day culture period of parental ZR-75-1 cells, vector controls and eight independently derived

Fig. 7. *BCAR3* induces antiestrogen resistance. (A) ZR-75-1: cell lines containing the expression vector without the *BCAR3* cDNA, *BCAR3* transfectants and parental ZR-75-1 cells (0.7×10^6) were plated in culture medium with 10% BCS and $1 \mu\text{M}$ 4-hydroxy-tamoxifen in triplicate. Cell numbers were determined on day 11. Cell lines indicated are: parental ZR-75-1 cells (ZR), LNCX (average of four different cell clones) and LXSN vector controls and the *BCAR3* transfectants (C4, C5, C9 and C10 under control of a CMV promoter and L6, L24, L31 and L33 under control of the LTR promoter). (B) MCF-7: pools of MCF7/*BCAR3* transfectants, MCF7/LNCX vector control transfectants and parental MCF-7 cells were plated in medium with 10% FCS with 100 nM of the pure antiestrogen ICI 182,780 in triplicate. Cells were counted at the indicated days and replated at the initial density of 0.25×10^6 cells per 25 cm^2 flask. Cumulative cell numbers are presented over a 50 day culture period.

transfectants is presented. Parental ZR-75-1 cells, which are fully dependent on estradiol for proliferation, give rise to maximally one generation in the presence of 4-hydroxy-tamoxifen (Figure 7A). As expected, similar results were obtained with the LNCX and LXSN vector controls. In contrast, the ZR/*BCAR3* cells have acquired the ability to proliferate in the presence of 4-hydroxy-tamoxifen. In addition, the proliferation capacity of the transfectants is

similar in the presence or absence of antiestrogens and thus not dependent on antiestrogens. Subcultivation after the 11 day culture period of ZR-75-1 cells and transfectants with the vector alone, in the presence of 4-hydroxy-tamoxifen, resulted in rapid decline of these cultures. Transfectants C5, C9, C10 with *BCAR3* under control of the CMV promoter were subcultured successfully and became expanding cultures. Cell clone L24 of the *BCAR3* transfectants with the LTR promoter only developed as a stable estrogen-independent cell line. The reason for these variations are at present not clear, but may be attributed to differences in *BCAR3* protein levels. In total, 40 transfectants were tested and 25 (62.5%) showed objective growth in the presence of 4-hydroxy-tamoxifen. Similar results were obtained with the antiestrogen ICI 182,780 a pure antagonist of estrogen-stimulated proliferation. None of the vector control cell lines or the parental ZR-75-1 cells, which were tested repeatedly, were able to grow in the presence of 4-hydroxy-tamoxifen or ICI 182,780.

Additional experiments were performed to establish whether *BCAR3* can induce antiestrogen resistance in the ER-positive MCF-7 breast cancer cell line. MCF-7 cells were transfected with *BCAR3* under control of the CMV promoter (MCF7/*BCAR3*) or with the LNCX vector without the insert (MCF7/LNCX). Both pools of stably transfected cells and individually established clones were obtained in medium containing 10% fetal calf serum (FCS) and G418. In total, 20 MCF7/*BCAR3* clones and eight MCF7/LNCX vector clones were isolated and tested for proliferation ability in the presence of the pure antiestrogen ICI 182,780. Fourteen out of 20 MCF7/*BCAR3* clones (70%) showed objective growth compared with the MCF7/LNCX clones. Similar results were obtained with cultures in the presence of 4-hydroxy-tamoxifen. Growth curves of the pools of transfectants and the parental MCF-7 cells in the presence of antiestrogen are presented in Figure 7B, and demonstrate the requirement for *BCAR3* for antiestrogen-resistant proliferation.

Discussion

Treatment failure of progressive breast cancer is inevitably due to the unremitting progression of the tumor cells to a more malignant hormone-independent phenotype. Insight into the different signaling pathways of normal and breast cancer cells leading to proliferation is a prerequisite for future development of new treatment strategies. *BCAR3*, a novel gene involved in progression of breast cancer cells to estrogen independence, was identified using retroviral insertional mutagenesis *in vitro*. A panel of 80 antiestrogen-resistant cell clones was screened for new common integration sites. In six cell clones, a proviral integration was detected in a restricted region. All viruses were integrated in an intron located in the 5' region of the *BCAR3* gene. Somatic cell hybrids between estrogen-dependent ZR-75-1 cells and one of the estrogen-independent *BCAR3* integration clones were established. Transfer of the *BCAR3* chromosomal locus resulted in immediate induction of antiestrogen resistance of the cell hybrids. Most likely as a consequence of the proviral integrations, a truncated protein lacking the N-terminal part of the protein is expressed in these cells. It was determined by Northern analysis that the large transcripts in the *BCAR3*

integration clones consisted of *BCAR3*, LTR and NeoR sequences. In addition, shorter *BCAR3* mRNAs, lacking the first exon(s), were observed in these cells. The shorter *BCAR3* product may originate from readthrough transcripts initiated by the viral 5' LTR (Kung *et al.*, 1991; Jonkers and Berns, 1996). These chimeric transcripts could be spliced from the splice donor site preceding the *gag* sequences (Miller and Rosman, 1989) in the retrovirus to the *BCAR3* exon sequences adjacent to the integration site. Translation of the resulting mRNAs may initiate at the ATG codon at position 436 and would result in a smaller protein. However, this truncation of *BCAR3* is not a prerequisite for the induction of the hormone-independent phenotype. Transfection of the *BCAR3* cDNA to estrogen-dependent ZR-75-1 and MCF-7 cells resulted in bypassing of hormone dependence and immediate antiestrogen resistance. These results suggest that up-regulation of *BCAR3* stimulates an alternative growth path independently of hormone, both in the presence and absence of antiestrogens. In the established cell lines studied, derived from the breast, ovary and endometrium, an inverse relationship between expression of *BCAR3* mRNA and ER protein was observed (McNemar test, $P = 0.025$). *BCAR3* expression was not detected in ER-positive cell lines. The strongest predictor for response to tamoxifen is expression of a functional ER in primary breast tumors (Foekens *et al.*, 1994; Spyrtos *et al.*, 1994; Johnston *et al.*, 1995), while EGF receptor expression has been associated with lack of response to endocrine therapy (Nicholson *et al.*, 1988, 1989). In the panel of breast-derived cell lines studied so far, EGF receptor expression is not tightly linked to *BCAR3* expression.

Sequence similarity analysis showed that *BCAR3* contains a copy of a putative SH2 protein domain. SH2 protein domains are found within a wide variety of cytoplasmic signaling molecules that can bind protein sequences phosphorylated at tyrosine residues (Cohen *et al.*, 1995; Pawson, 1995). The homology of *BCAR3* with other SH2 domain-containing proteins is limited to this domain. The amino acid residues implicated in protein structure and in interaction with the phosphotyrosine are well conserved. Major sequence differences are observed in the amino acid sequences spacing the β -sheets and α -helices as well as residues implicated in the interaction with substrate amino acid residues adjacent to the phosphorylated tyrosine (Cohen *et al.*, 1995). The *BCAR3* SH2 domain shows the strongest homology to SH2 domains of Shc, Grb2, Fer and Fes. In analogy to these related proteins, *BCAR3* may act as an adaptor protein and couple activated growth factor receptors to a signaling pathway that regulates the proliferation in these breast cancer cells. Its substrate specificity may resemble that of the Grb2 protein (Songyang *et al.*, 1994), based on the conservation of the critical residues (Figure 4C, marked with #) in the β D region, and indicates a link to the Ras signaling cascade (Rozakis-Adcock *et al.*, 1992).

BCAR3 protein displays striking partial homology with a cell division cycle gene product (CDC48) from yeast. Mutation or elimination of CDC48 in yeast results in arrest of cell division in the large budded state with an undivided nucleus and unseparated spindle pole body (Fröhlich *et al.*, 1991). The characteristic feature of this protein is an internally duplicated domain for nucleotide

binding, which is conserved in its mammalian homolog the valosin-containing protein (VCP). ATPase activity has been demonstrated for these proteins (Schulte *et al.*, 1994; Fröhlich *et al.*, 1995). CDC48 proteins shows significant homology to a group of NSF-like proteins which also comprise the duplicated ATPase domain and are involved in homeotypic fusion of intracellular membranes (Fröhlich *et al.*, 1991; Acharya *et al.*, 1995; Latterich *et al.*, 1995; Mellman, 1995; Rabouille *et al.*, 1995). None of the domains shared between CDC48 and the NSF-like proteins are conserved in the *BCAR3* protein. *BCAR3* has acquired the C-terminal part of CDC48 proteins, which has not yet been implicated in a specific function and includes ~30 amino acid residues contained within the duplicated domain. The mammalian CDC48 is strongly phosphorylated on tyrosine and serine residues in B and T cells and in v-Src transformed fibroblasts (Egerton *et al.*, 1992; Schulte *et al.*, 1994). Primarily from *in vitro* experiments with T cells it was concluded that the C-terminal Tyr805 in murine CDC48 is the predominant target for phosphorylation, but phosphorylation of this residue is non-essential for ATPase function (Egerton and Samelson, 1994). This particular tyrosine residue is not conserved in *BCAR3* nor in the archaeobacterial homolog of CDC48 (Figure 4D, Sa). An adjacent tyrosine residue is conserved in CDC48 proteins of all species tested and in the *BCAR3* protein, and represents an attractive target for C-terminal phosphorylation and functional modulation of these proteins.

The *BCAR3* protein has characteristics of a molecule involved in signal transduction. The putative SH2 domain is most likely involved in binding of tyrosine-phosphorylated proteins and may represent an essential component of cascades transporting signals from the cell membrane to the nucleus. The C-terminal domain of *BCAR3* is homologous to the CDC48 protein which is responsible for cell division control in yeast and subject to tyrosine phosphorylation in mammalian cells. The combination of these features may explain its dominant role in antiestrogen-arrested breast cancer cell proliferation upon over-expression by retroviral insertion or ectopic expression. Future experiments will attempt to delineate the contribution of these components and define the signaling pathway involved. Further work will be required to establish whether *BCAR3* is involved in clinical breast cancer and possibly in other endocrine-related tumors.

Materials and methods

Cell lines and culture conditions

Cell lines were obtained from the American Type Culture Collection (Rockville, MD), except for ZR-75-1 and MCF-7 which were kind gifts of R.J.B.King (ICRF, London) and R.B.Dickson (NCI, Bethesda) respectively. Cell culture was performed essentially as described (Van Agthoven *et al.*, 1992, 1994; Dorssers *et al.*, 1993; Sieuwerts *et al.*, 1997). ZR-75-1, ZH3D7, hybrid and ZR/*BCAR3* cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum (BCS) and 1 nM 17 β -estradiol. *BCAR3* integration cell clones were maintained in RPMI 1640 medium supplemented with 15% BCS, 10% conditioned medium of CRIP cells (a mouse fibroblast cell line) and 1 μ M 4-hydroxy-tamoxifen. MCF-7 and MCF7/*BCAR3* cells were cultured in RPMI 1640 with 10% heat-inactivated FCS.

Cell fusion

Cell fusion experiments were performed as described (Dorssers and Veldscholte, 1997). Briefly, ZR-75-1 cells were transfected with the expression construct PGK-Hyg B^r which confers hygromycin B resist-

ance, and were used as acceptor cells (designated ZH3D7). Clone XI-1-6A, carrying two viral integrations, was selected as donor cell line. As a consequence of the retroviral insertions, these cells are both antiestrogen- and G418-resistant. Furthermore, the 4-hydroxy-tamoxifen-resistant cells were lethally irradiated to prevent outgrowth of the donor cells. ZH3D7 and XI-1-6A cells were fused with polyethylene glycol (PEG, Boehringer Mannheim, Germany) using a procedure for adherent cells (Eijdens *et al.*, 1992) and according to the manufacturer's recommended conditions. After fusion, the cells were allowed to recover for 2 days and subsequently selected with 1 mg/ml G418 (Geneticin, Gibco-BRL, Life Technologies Ltd, Paisley, UK) in the presence of 1 nM 17 β -estradiol. After 10 days, the selection was continued with 500 μ g/ml G418 and 25 μ g/ml hygromycin B (Boehringer Mannheim) until clones developed. Clones were picked, expanded, characterized by Southern analysis and challenged for antiestrogen resistance.

Transfection of expression constructs

A near full-length BCAR3 cDNA (#32, nucleotides 39–2989) was cloned into the pLXSN and pLNCX expression vectors (Miller and Rosman, 1989) in which transcription is driven in the former by the LTR promoter and in the latter by a CMV promoter. Expression constructs and control vectors without inserts were transfected using lipofectin reagent (Life Technologies). ZR-75-1 cells (2×10^5) were seeded in 25 cm² flasks (Costar, Cambridge, MA) in medium containing 17 β -estradiol. After 2 days, the cells were washed twice with serum-free Optimem medium (Life Technologies). To the cells, 3 ml of Optimem medium with 30 μ l of lipofectin was added. After 1 h incubation at 37°C, 2–5 μ g of linearized plasmid DNA was added. Following 5 h incubation, the DNA-containing medium was replaced by RPMI medium, containing 10% BCS and 1 nM 17 β -estradiol. Subsequently, after 2 days, selection with G418 was started. Individual transfectants were isolated and propagated in medium with estradiol and G418. MCF-7 cells were transfected similarly with pLNCX with and without insertion in RPMI medium containing 10% FCS.

DNA and RNA analysis

Genomic DNA was isolated using NaCl extraction procedures described by Miller *et al.* (1988). Total RNA was isolated using guanidine/cesium chloride extraction and used for Northern analyses as previously described (Van Agthoven *et al.*, 1994). Probes were random-primed labeled using [α -³²P]dATP (ICN Pharmaceuticals Inc. CA). Blots were exposed to X-OMAT AR film (Eastman Kodak Company, NY).

Isolation and sequencing of BCAR3 cDNA clones

A testis cDNA library (Clontech Laboratories, Inc., CA) was screened with the BCAR3 integration-specific probe. Plaques were purified and the cDNA inserts were recloned in pGEM4Z (Promega, Madison, WI). The nucleotide sequence was determined on both strands by dideoxy sequencing reactions using T7 DNA polymerase (Pharmacia Biotech, Sweden) and [α -³²P]dATP (ICN Pharmaceuticals Inc.). 5' RACE experiments were performed according to the manufacturer's recommendations (Boehringer Mannheim).

In vitro translation

The near full-length BCAR3 cDNA clone #32 was recloned in the EcoRI site of pGEM4Z. A shortened construct (nucleotides 421–2989) resembling the alteration in the BCAR3 clones was obtained by subcloning the cDNA clone #32. The plasmid was transcribed from the T7 promoter and translated in rabbit reticulocyte lysate by using the TNT Coupled Reticulocyte Lysate System (Promega). After SDS-PAGE, recombinant proteins were electrotransferred to nitrocellulose membranes and proteins were detected with the Non-Radioactive Translation Detection System (Promega) and chemiluminescence (Amersham International, Bucks, UK) by exposing to X-OMAT AR film.

Growth assays

ZR-75-1 cells and transfectants cultivated in the presence of 1 nM 17 β -estradiol were harvested by treatment with trypsin-EDTA. Single cells (0.7×10^6) were plated in 25 cm² plastic culture flasks in triplicate. The experimental medium containing 10% BCS and 1 μ M 4-hydroxy-tamoxifen was changed twice a week. After 11 days, the cultures were harvested by trypsinization and counted. Subsequently, 1.5×10^6 cells were replated to determine the secondary growth rate. MCF-7 cells and transfectants routinely were cultured in medium containing 10% FCS. Cells (0.25×10^6) were plated in experimental medium with 10% FCS and 100 nM ICI 182,780, harvested at days 8 and 11, and replated at the initial density to determine the secondary growth rate.

Receptor determinations

ER and progesterone receptor were determined as described (Van Agthoven *et al.*, 1992), using commercially available enzyme immunoassay kits (ER-EIA, PR-EIA; Abbott Laboratories, Chicago, IL). EGF receptors were determined using a commercially available enzyme-linked immunosorbent assay kit (Oncogene Science, Cambridge, MA).

Nucleotide accession number

The sequence data reported here have been submitted to the DDBJ/EMBL/GenBank database under accession number U92715.

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