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Journal of Cellular Biochemistry 105:866–874 (2008)

Journal of Cellular Biochemistry

Rcl Is a Novel ETV1/ER81 Target Gene Upregulated in Breast Tumors

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ABSTRACT

ETV1 (ER81) is a transcription factor that can be activated by HER2/Neu, a proto-oncoprotein often overexpressed in metastatic breast tumors. Here, we demonstrate that ETV1 downregulation suppresses proliferation of HER2/Neu-positive MDA-MB-231 breast cancer cells in vitro and tumor formation in vivo, proving for the first time the existence of a critical role of ETV1 in breast cancer cell physiology. A screen for novel ETV1 target genes hinted at Rcl, an enzyme involved in nucleotide metabolism. To characterize the human Rcl gene, we cloned its promoter and found that ETV1 and HER2/Neu cooperated in activating the Rcl promoter, whereas a dominant-negative ETV1 molecule suppressed the Rcl promoter. Moreover, ETV1 and HER2/Neu synergized to upregulate the endogenous Rcl gene. ETV1 also bound to the Rcl promoter in vivo, indicating that Rcl is a bona fide target gene of ETV1. Hybridization of Rcl cDNA to a breast cancer array revealed that Rcl is overexpressed in ~40% of all breast tumors. Importantly, its expression significantly escalates with increasing tumor grade, strongly implicating that Rcl contributes to breast tumorigenesis. Since joint overexpression of Rcl with vascular endothelial growth factor, another target gene of ETV1, has been shown to induce tumor formation, Rcl may be one crucial effector of ETV1 and HER2/Neu in breast tumors. Furthermore, given its expression pattern and enzymatic function in nucleotide metabolism, Rcl presents itself as a novel target in breast cancer therapy via modulation of its activity by small molecule drugs. J. Cell. Biochem. 105: 866–874, 2008.

KEY WORDS: BREAST CANCER; ER81; ETV1; HER2/NEU; RCL

ETS variant 1 (ETV1), also called ER81 (ETS related 81), is a transcription factor that is a member of the ETS family of DNA binding proteins [Brown and McKnight, 1992; Janknecht and Nordheim, 1993]. Its association with cancer was first noted over a decade ago in Ewing tumors, in which the EWS gene can be translocated onto the ETV1 gene and the resultant EWS-ETV1 fusion protein exerts oncogenic properties [Jeon et al., 1995; Janknecht, 2005]. Notably, the EWS-ETV1 fusion protein is a chimeric transcription factor that consists of the potent N-terminal EWS transactivation domain and the C-terminus of ETV1 encompassing its ETS DNA binding domain. As such, since the genuine ETV1 protein contains only weak activation domains that require stimulation to become effective [Janknecht, 1996; Coutte et al., 1999], the EWS-ETV1 protein represents a constitutively activated ETV1 molecule capable of inappropriately upregulating ETV1 target genes. In addition, recurrent chromosomal translocations have recently been found in prostate cancer leading to the overexpression of ETV1 [Tomlins et al., 2005, 2007]. These findings suggest that dysregulation of ETV1 target genes in disparate tumors like Ewing sarcomas and prostate carcinomas is causally involved in tumorigenesis and point to a general importance of ETV1 target genes in cancer.

Unfortunately, only few ETV1 target genes are known. These include matrix metalloproteinase-1 and -7 and heparanase, enzymes that promote tissue invasion and metastasis [Bosc et al., 2001; Crawford et al., 2001; Fuchs et al., 2003; Lu et al., 2003]; vascular endothelial growth factor (VEGF), one of the most important drivers of angiogenesis in tumors [Fuchs et al., 2004b]; telomerase reverse transcriptase, the catalytic subunit of telomerase whose upregulation is responsible for the immortalization of over 90% of all tumor cells [Goueli and Janknecht, 2004; Janknecht, 2004; Fuchs et al., 2004a]; Smad7, an inhibitor of the tumor suppressing activity of the transforming growth factor-β signaling pathway [Dowdy et al., 2003]; and HER2/Neu, a receptor tyrosine kinase and proto-oncoprotein especially associated with breast cancer [Bosc and Janknecht, 2002; Holbro et al., 2003]. Of note,

Grant sponsor: Department of Defense Breast Cancer Research Program; Grant number: DAMD17-03-1-0538; Grant sponsor: Fraternal Order of Eagles Cancer Research Fund; Grant sponsor: Breast Cancer Research Foundation; Grant sponsor: Bruce and Martha Atwater Foundation.

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Received 25 May 2008; Accepted 15 July 2008 • DOI 10.1002/jcb.21884 • 2008 Wiley-Liss, Inc.

Published online 22 August 2008 in Wiley InterScience (www.interscience.wiley.com).

ETV1 transcriptional activity is dramatically enhanced upon HER2/Neu overexpression [Bosc et al., 2001; Goel and Janknecht, 2003], suggesting the existence of a feed-forward loop in the upregulation of HER2/Neu.

Moreover, ETV1 mRNA levels are increased in murine cell lines and tumors overexpressing HER2/Neu [Shepherd et al., 2001; Galang et al., 2004] and also in many human breast cancer cell lines [Baert et al., 1997], suggesting that ETV1 may contribute to breast tumorigenesis. Although transgenic mice overexpressing ETV1 in the breast do not develop mammary tumors [Netzer et al., 2002], ETV1 overexpression may prime breast cells to become malignant, for instance upon additional overexpression of HER2/Neu. In support of such a priming function for ETV1, its overexpression in the prostate of transgenic mice induced the formation of the precursor of prostate cancer, namely prostatic intraepithelial neoplasia, but not metastatic carcinomas [Tomlins et al., 2007].

To gain more insight into ETV1's role in breast tumorigenesis, we attempted to identify additional ETV1 target genes. Here, we report that Rcl, originally cloned as a c-Myc inducible gene capable of transforming Rat1a fibroblasts [Lewis et al., 1997; Fang et al., 1999; Kim et al., 2003; Zeller et al., 2003], is a novel ETV1 target gene potentially implicated in the genesis of breast cancer.

MATERIALS AND METHODS

DOWNREGULATION OF ETV1

Two sequences in the coding region of human ETV1 (#1: GTGCCTGTACAATGTCAGT; #2: GGGAAGGACGTCCTATGTA) were targeted by shRNAs. Corresponding oligonucleotides were cloned into the lentiviral vector pLentiLox3.7. Virus was produced according to standard procedures [Shin and Janknecht, 2007a] and utilized to infect MDA-MB-231 cells. Cell proliferation was monitored with a cell counting kit 8 (Alexis) by measuring absorbance of WST-8 formazan at 450 nm. Alternatively, 5×10^6 MDA-MB-231 cells, in which ETV1 was downregulated, were injected into the left flank of female, 6-week-old athymic NCr-*nu/nu* nude mice and tumor mass determined 63 days afterwards. This experiment was approved by the Mayo Clinic Institutional Animal Care and Use Committee.

TOGA

HA-VP16-ETV1, in which an HA-tag and the VP16 transactivation domain were fused to murine ETV1 amino acids 314–477, was stably transfected into U2OS cells. RNA was extracted from these cells and control cells transfected with the empty expression vector and then employed for total gene expression analysis (TOGA) essentially as described [Sutcliffe et al., 2000].

RT-PCR

Cytoplasmic RNA was isolated from 293T cells transiently transfected in 6-cm dishes as described before [Bosc et al., 2001]. 0.5 μg of ETV1, 1 μg of HER2/Neu-V664E [Ben-Levy et al., 1994], or 0.2 μg of EWS-ETV1 or HA-VP16-ETV1 expression vector were employed for transfection. Approximately 0.5 μg of RNA was then utilized for the RT-PCR reaction employing the Access RT-PCR kit (Promega). Rcl was amplified with primers 5′-GCTCATCCATGAG-CAGGACCTG-3′ and 5′-GCTAAGAGGAAGGAATGGTACTAG-3′ under the following conditions: 48°C for 45 min, 96°C for 2 min, 32 cycles of 94°C for 30 s, 58°C for 45 s and 68°C for 2 min, followed by a 5 min long incubation at 68°C. GAPDH was amplified with primers 5′-GAGCCACATCGCTCAGACACC-3′ and 5′-TGACAAG-CTTCCCGTTCTCAGC-3′ as described before [Goel and Janknecht, 2003]. DNA was electrophoresed on 1.5% agarose gels and the 371 bp Rcl and the 226 bp GAPDH cDNA fragments were visualized by ethidium bromide staining [Shin et al., 2007].

HYBRIDIZATIONS

A human polyA$^+$ Northern blot (Clontech) was hybridized with ^{32}P-labeled human Rcl cDNA derived as a 0.55 kb *Xho*I/*Xba*I fragment from HA-hRcl expression vector. Hybridization of the Northern blot and washing procedures were according to standard procedures [De Haro and Janknecht, 2005]. Similarly, a breast cancer profiling array (Clontech) or a cancer cell line profiling array (Clontech) was hybridized with ^{32}P-labeled human Rcl cDNA or cDNA coding for the first 246 amino acids of human EWS according to the manufacturer's recommendations.

RACE AND Rcl PROMOTER CLONING

The Rcl transcription start and polyadenylation sites were identified with the help of the GeneRacer Kit (Invitrogen) utilizing polyA$^+$-mRNA from human kidney (Clontech). For 5′-RACE (rapid amplification of cDNA ends), we employed random primers or an Rcl exon 3 primer (5′-CACCCAAGGATGGCTGTGTCACTTCTG-3′) in the reverse transcriptase reaction. Afterwards, a first PCR was performed with an Rcl exon 2 primer (5′-CCACTCCAGGTCCTGCT-CATGGATGAG-3′) and the GeneRacer-5′ primer, followed by a second PCR with the Rcl exon 2 primer and the GeneRacer-5′-Nested primer. PCR products were separated on an agarose gel, DNA extracted from respective gel slices, cloned into the pCR-BluntII-TOPO (Invitrogen) vector and the nature of the PCR products then determined by DNA sequencing. In the case of 3′-RACE, we utilized the GeneRacer-Oligo(dT) primer for reverse transcription, and PCR was performed with the GeneRacer-3′ primer and the Rcl specific primer 5′-CAAGCGGATCCTGTGCCTGTTCCG-3′.

Rcl DNA encompassing 1,273 bp upstream and 204 downstream of its transcription start site was amplified by genomic PCR. The resulting PCR product was designed to have a *Kpn*I or *Hin*dIII site at the upstream or downstream end, respectively, and was cloned into *Kpn*I/*Hin*dIII-cut pGL2-Basic (Promega). Shorter Rcl promoter fragments were inserted into pGL2-Basic utilizing standard cloning procedures.

IMMUNOSTAINING

Cells were grown on coverslips and transiently transfected with 6Myc-hRcl expression vector similarly as described before [Rossow and Janknecht, 2001]. Thirty-six hours later, cells were fixed with formaldehyde and stained with anti-Myc 9E10 mouse monoclonal antibody [Knebel et al., 2006].

LUCIFERASE ASSAYS

Mv1Lu cells grown in 12-wells were transiently transfected by the calcium phosphate coprecipitation method [Janknecht et al., 1993;

Shin and Janknecht, 2007c]. Per well, 200 ng of luciferase reporter plasmid, 300 ng of ETV1 expression vector or empty vector pEV3S, 30 ng of HER2/Neu-V664E expression vector and 2 μg of pBluescript KS^+ as a carrier were employed for transfection [Kim et al., 2008]. Thirty-six hours after transfection, cells were lysed in 25 mM Tris–HCl (pH 7.8), 2 mM EDTA, 10% glycerol, 1% Triton X-100 and 2 mM DTT [Rossow and Janknecht, 2003]. Debris was removed by centrifugation and the supernatant employed to measure luciferase activity as described before [Janknecht and Hunter, 1997; De Haro and Janknecht, 2002].

ChIP ASSAYS

Chromatin immunoprecipitation (ChIP) assays were essentially performed as previously described [Goueli and Janknecht, 2003; Shin and Janknecht, 2007b]. The following primers were utilized to amplify a 394 bp fragment of the human Rcl promoter: 5′-CGA-CATGTGGACACCTGTTAGGAG-3′ and 5′-AGTCCACACCTGTGG-AATGGGGAC-3′. The primer pair 5′-GTGGCTCTGATTGGCTTT-CTG-3′ and 5′-CCAGCCCTGTCGCAAGGATC-3′ was employed to amplify a 218 bp fragment of the human p21 promoter. The PCR program utilizing iProof high fidelity DNA polymerase (BioRad) was: 98°C for 2 min, 10 cycles of 98°C for 30 s, 65°C for 30 s (minus 1°C per cycle), 72°C for 20 s followed by 26 cycles of 98°C for 30 s, 55°C for 30 s, 72°C for 20 s (plus 1 s per cycle) and finally by a 5 min extension at 72°C.

RESULTS

ETV1 ENHANCES BREAST CANCER CELL PROLIFERATION

Hitherto, a role of ETV1 in breast cancer has only been inferred from its overexpression pattern in breast cancer cells and its ability to become stimulated by and thereby potentially mediating the effects of the HER2/Neu oncoprotein. To prove ETV1's importance for breast cancer more directly, we elected to study the role of endogenous ETV1 in the breast cancer cell line MDA-MB-231, which expresses low levels of endogenous HER2/Neu [Hollywood and Hurst, 1993]. Two different shRNAs expressed by lentivirus were employed to knock-down endogenous ETV1 expression. As shown in Figure 1A, both shRNAs efficiently downregulated ETV1 expression. Importantly, proliferation of MDA-MB-231 cells was dramatically reduced upon downregulation of ETV1 (Fig. 1B). Moreover, downregulation of ETV1 also diminished the ability of MDA-MB-231 cells to form tumors in athymic mice (Fig. 1C). Collectively, these data indicate that ETV1 promotes proliferation of MDA-MB-231 cells, emphasizing that ETV1 can be crucial for breast cancer cell physiology.

IDENTIFICATION OF A NOVEL ETV1 TARGET GENE

To better understand how ETV1 may contribute to the causation of breast cancer, one has to determine the changes of the transcriptome that are caused by dysregulation of ETV1. A hurdle in readily identifying such changes is that ETV1 in itself is nearly inactive and can even inhibit gene transcription at certain gene promoters in the absence of prior stimulation through protein kinases [Janknecht, 1996; Coutte et al., 1999]. Therefore, to circumvent this obstacle, we designed a chimeric protein (VP16-ETV1) in which the potent transactivation domain of the herpex simplex virus regulatory protein VP16 was fused to the C-terminus of ETV1 encompassing its DNA binding domain, thereby creating a constitutively active form of ETV1 that resembles the EWS-ETV1 fusion protein found naturally in Ewing tumors. VP16-ETV1 was stably transfected into U2OS cells and changes in gene expression monitored with the TOGA procedure [Sutcliffe et al., 2000]. One potential ETV1 target gene thereby identified was Rcl, a scarcely studied gene capable of transforming fibroblast cells that was recently shown to encode for an enzyme involved in nucleotide metabolism [Lewis et al., 1997; Ghiorghi et al., 2007].

To confirm that Rcl transcription is upregulated by ETV1, we expressed VP16-ETV1, EWS-ETV1, or ETV1 in the absence or presence of HER2/Neu in 293T cells. RNA was isolated from these cells and subjected to RT-PCR to assess the levels of Rcl mRNA. Indeed, both VP16-ETV1 and EWS-ETV1 strongly stimulated Rcl mRNA expression compared to the control (Fig. 2), while overexpression of either ETV1 or HER2/Neu alone did not elicit Rcl expression. Strikingly, when ETV1 was stimulated by coexpression of HER2/Neu, robust Rcl expression was observed (Fig. 2). These data

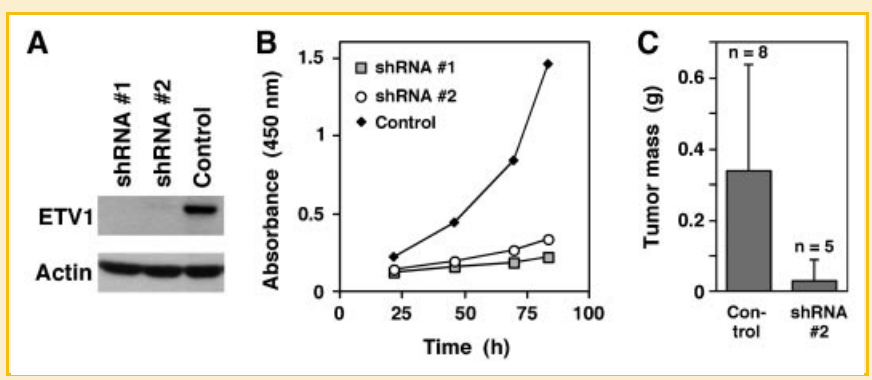

Fig. 1. ETV1's role in breast cancer cell proliferation. A: Downregulation of ETV1 by two different shRNAs in MDA-MB-231 cells. Shown are Western blots of ETV1 and actin as a control. B: MDA-MB-231 cells expressing control or ETV1 shRNAs were plated at equal density and proliferation (indicated by absorbance at 450 nm) monitored over the next 3.5 days. C: Equal numbers of MDA-MB-231 cells expressing control or ETV1 shRNA#2 were injected into athymic mice and tumor mass determined after 63 days.

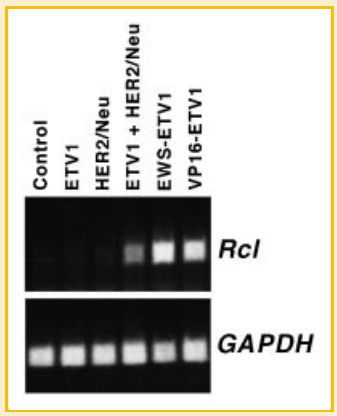

Fig. 2. 293T cells were transfected with indicated expression plasmids. RT-PCR was performed to reveal Rcl mRNA expression. GAPDH mRNA levels were determined as a control.

indicate that Rcl is a downstream target of ETV1. The next step was to determine whether Rcl is a direct target gene of ETV1 or indirectly upregulated by ETV1. To address this point, we first needed to clone the human Rcl gene and its promoter.

CLONING OF THE HUMAN Rcl GENE

To characterize the Rcl gene, we initially determined its expression in multiple human tissues by Northern blotting. Rcl was highly expressed in kidney, liver and skeletal muscle, moderately expressed in the heart and spleen, and scarcely expressed in brain, colon, thymus, small intestine, placenta, lung and peripheral blood leukocytes (Fig. 3A). We also assessed the expression of Rcl in several cancer cell lines grown under low or high serum conditions (Fig. 3B). High expression of Rcl was noted in MCF7 but not MDA-MB-435S or MDA-MB-231 breast cancer cells, in DU145 but not PC-3 prostate cancer cells, in SK-MEL-5 but not SK-MEL-28 melanoma cells, and in SK-N-SH and IMR-32 but not U-87 MG brain cancer cells. These results indicate that Rcl expression might be upregulated in some cancer cell lines, including those derived from breast tumors.

Since Rcl was most highly expressed in the kidney, we utilized human kidney polyA$^+$-mRNA to determine the boundaries of Rcl cDNA employing the RACE procedure. We found through DNA sequencing of 17 5′-RACE clones that transcription most often (8 clones) starts at the nucleotide designated +1 in Figure 4; less frequently, transcription started at +4 (5 clones) or at +2, +3, +6, and +20 (one clone each). On the other hand, DNA sequencing of 19 3′-RACE clones revealed that attachment of polyA-tails occurred in 18 cases at +654 and only in one case at +648. We are confident that the sequence from +1 to +654 represents full-length Rcl mRNA, since 654 nucleotides plus the average length of polyA-tails of 100–200 nucleotides matches very well the length of Rcl mRNA determined by Northern blotting to be ~800 nucleotides (see Fig. 3A).

Analysis of full-length Rcl cDNA revealed an open reading frame starting at +26 and stopping at +550, encoding for a protein of 174 amino acids (Fig. 4). Notably, translation may not start with the

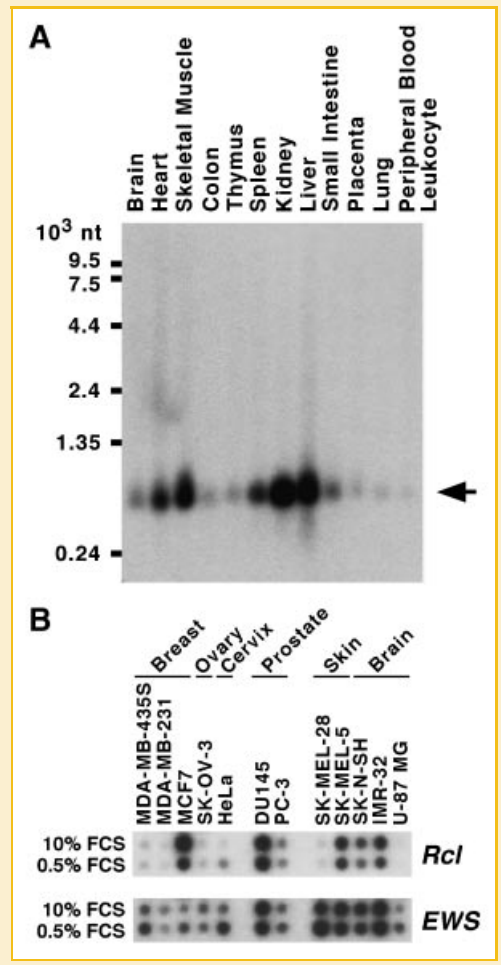

Fig. 3. A: Northern blot showing the expression of human Rcl mRNA in multiple tissues. Equal amounts of mRNA are present in all lanes. B: Hybridization of ^{32}P-labeled Rcl cDNA, or EWS cDNA for comparison, to a cancer cell line profiling array. Cancer cells from the indicated organs were cultured in 0.5% or 10% fetal calf serum (FCS).

methionine codon at +26, but rather with the methionine codon at +38, since this is embedded in a much more favorable Kozak sequence. Comparison to the previously reported 163 amino acids long rat Rcl protein [Lewis et al., 1997] and the theoretical 173 amino acids long murine Rcl protein revealed that human Rcl is 73.6% or 72.4% identical to rat or murine Rcl, respectively, while murine and rat Rcl are 86.1% identical (Fig. 5A). This demonstrates a high degree of evolutionary conservation of Rcl. Database searches indicated that amino acids 23–139 of human Rcl are 43% homologous to the nucleoside 2-deoxyribosyltransferase motif, which would be in agreement with the recent discovery that Rcl is an enzyme involved in nucleotide metabolism [Ghiorghi et al., 2007]. Moreover, we analyzed the intracellular localization of human Rcl. In two different breast cancer cell lines (Fig. 5B) as well as in cells derived from other organs (293T, Ovcar-3, RK13, Mv1Lu; data not shown), Rcl was predominantly localized within the cytoplasm (Fig. 5B), consistent with its suggested role in nucleotide metabolism.

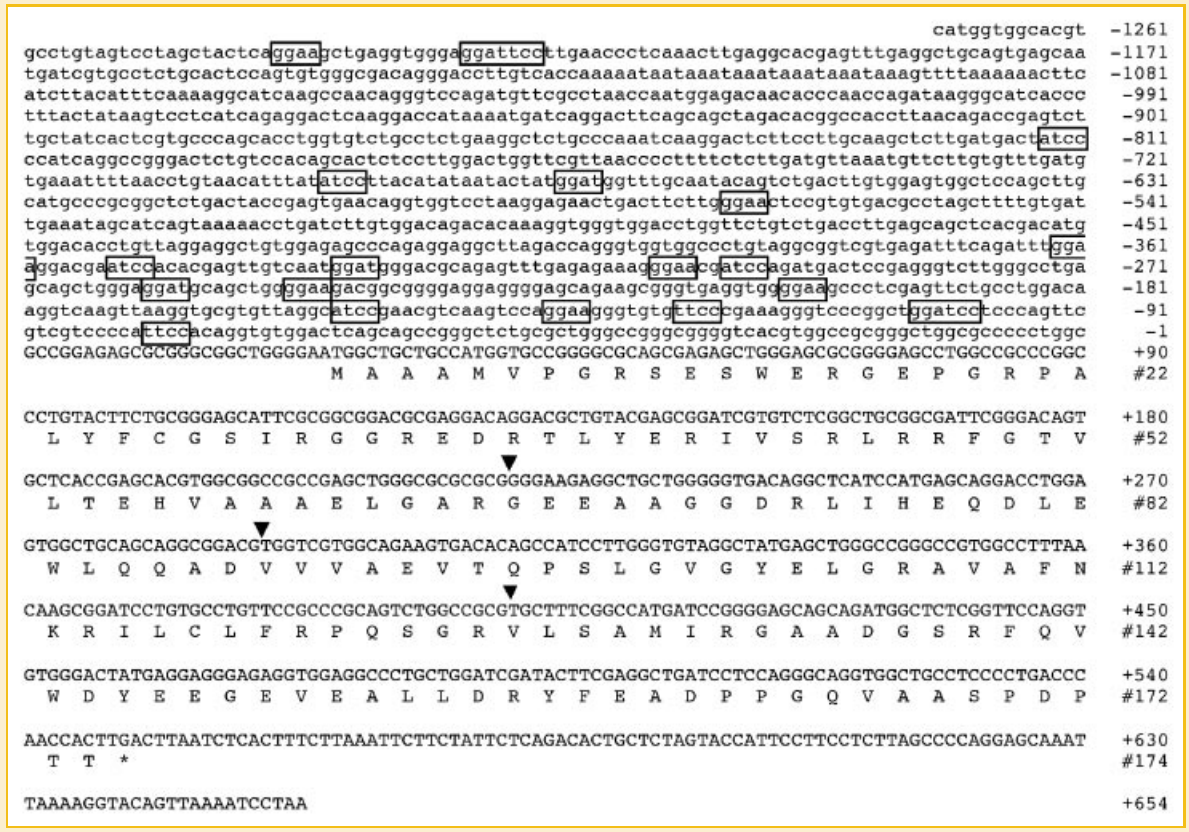

Fig. 4. Sequence of the Rcl upstream promoter and cDNA. Shown are 1,273 bp of promoter sequence (small letters) and 654 bp of human Rcl cDNA (capital letters; deposited in GenBank under accession number EU585603). The sequence of the deduced Rcl protein is indicated below the cDNA. Exon boundaries are indicated by triangles. Possible binding sites for ETS proteins ($GGA^{A}/_{T}$ or in reverse, $^{T}/_{A}TCC$) in the upstream promoter region are boxed.

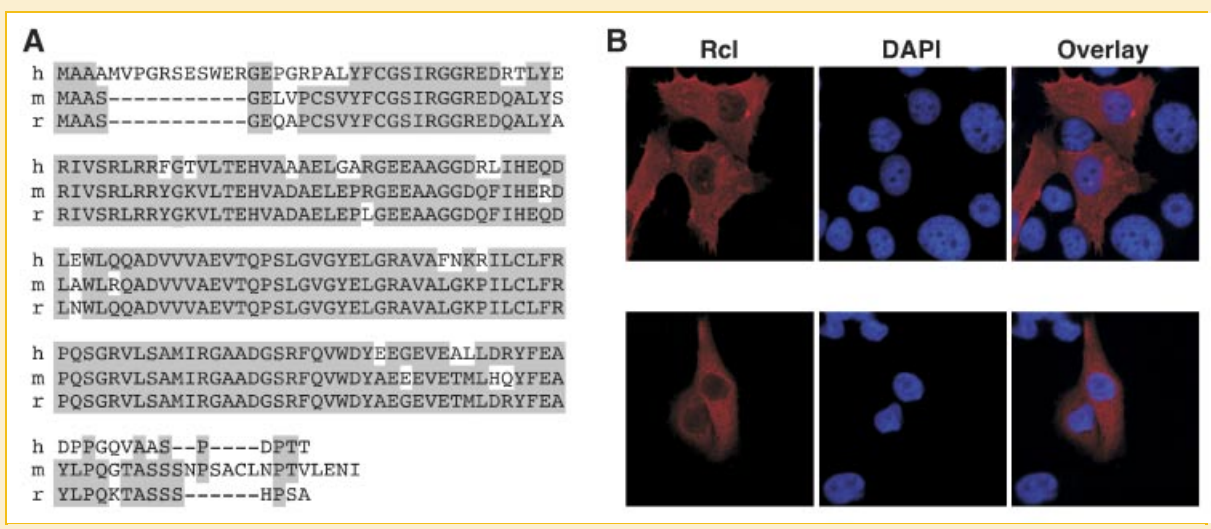

Fig. 5. The Rcl protein. A: Alignment of human (h), murine (m; NP_997044)) and rat (r; NP_598209) Rcl protein sequences. Identical amino acids are highlighted by shading. B: MDA-MB-231 (top) and Hs578T (bottom) breast cancer cells were transiently transfected with Myc-tagged Rcl expression vector and stained with anti-Myc antibody and TexasRed-coupled secondary anti-mouse antibody (left panels). DNA was additionally stained with DAPI (middle panels), and the right panels show a digital overlay of both stainings. Images were taken with a confocal laser microscope. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

REGULATION OF THE Rcl PROMOTER BY ETV1 AND HER2/Neu

Having defined the transcription start site of the human Rcl gene allowed us to clone its proximal promoter by genomic PCR. Thereby, we obtained a promoter fragment ranging from −1,273 upstream of the transcription start site to +204 within the first exon of the Rcl gene. Moreover, we obtained shorter Rcl promoter fragments (−387/+204, −252/+204, and −104/+204) and cloned all of these in front of the firefly luciferase gene. All these shorter Rcl promoter fragments were as active as the −1,273/+204 fragment (data not shown), indicating that nucleotides −104 to +204 are sufficient to mediate basal transcription of the Rcl promoter.

Analysis of the Rcl upstream promoter sequence revealed 21 ETS core sites (5′-GGA$^{A}/_{T}$-3′) that can potentially be bound by ETV1. Most of the ETS core sites are concentrated within 0.4 kb upstream of the transcription start site (see Fig. 4). To explore whether ETV1 binds to the Rcl promoter, we performed ChIP assays with MDA-MB-231 breast cancer cells. Utilizing primers that span the Rcl promoter from −457 to −64, we observed that anti-ETV1, but not control antibodies, immunoprecipitated Rcl promoter fragments (Fig. 6A). Furthermore, neither control nor anti-ETV1 antibodies immunoprecipitated a fragment of the p21 promoter. These results indicate that ETV1 binds to the Rcl promoter in vivo.

Next, we assessed if ETV1 can stimulate the −387/+204 and −1,273/+204 Rcl promoter fragments. Indeed, overexpression of ETV1 resulted in comparable, ∼2.5-fold stimulation of the corresponding luciferase promoter constructs (Figs. 6B and 7),

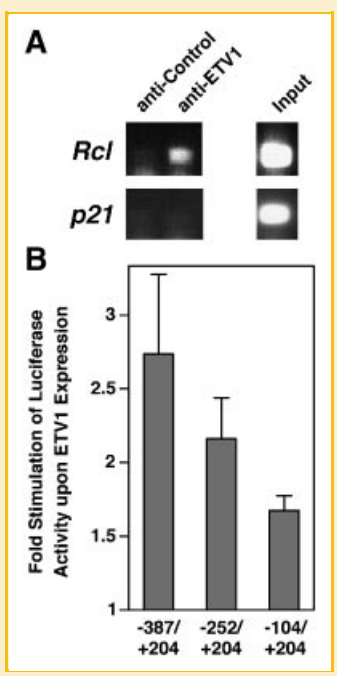

Fig. 6. Activation of the Rcl promoter by ETV1. A: ChIP assay showing binding of ETV1 to the human Rcl, but not p21 promoter. Promoter fragments were amplified by PCR and revealed by ethidium bromide staining of agarose gels. Input is reflective of total chromatin before immunoprecipitation with control or anti-ETV1 antibodies. B: Stimulation of promoter activity upon expression of ETV1 observed with the −387/+204, −252/+204, and −104/+204 Rcl luciferase reporter constructs.

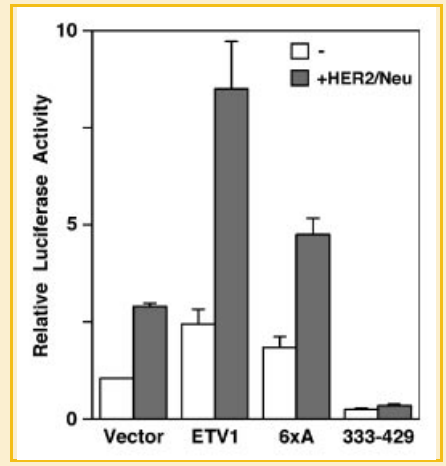

Fig. 7. A luciferase reporter construct driven by Rcl sequences −1,273/+204 was cotransfected with empty vector, wild-type ETV1, its phosphorylation mutant 6xA or $ETV1_{333-429}$ and luciferase activities determined 36 h thereafter. Where indicated, an expression plasmid for HER2/Neu was additionally transfected.

indicating that ETS sites upstream of −387 are not critical for ETV1-dependent Rcl promoter stimulation. However, successive truncation of the Rcl promoter from −387 to −252 and then to −104, thereby deleting more and more potential ETV1 binding sites, gradually reduced ETV1's ability to stimulate the Rcl promoter (Fig. 6B). This suggests that multiple ETV1 binding sites exist in the Rcl promoter that collaborate to mediate its inducibility by ETV1.

Like ETV1, overexpression of HER2/Neu on its own slightly induced Rcl promoter activity (Fig. 7). Importantly, joint expression of ETV1 and HER2/Neu strongly elevated luciferase activity. In contrast, expression of a 6xA mutant of ETV1, in which its HER2/Neu-inducible phosphorylation sites at pos. 94, 139, 143, 146, 191, and 216 are mutated to alanine [Bosc et al., 2001], still raised basal levels of luciferase activity, but expectedly showed decreased cooperation with HER2/Neu (Fig. 7). The residual cooperation of the 6xA mutant with HER2/Neu can be explained by the fact that ETV1 interacts with coactivators such as p300 and SRC-3/AIB1/ACTR that themselves are stimulated by HER2/Neu [Papoutsopoulou and Janknecht, 2000; Goel and Janknecht, 2004]. Indeed, ETV1 amino acids 333–429, which no longer interact with p300 and SRC-3/AIB1/ACTR but can still bind to DNA, were basically unresponsive to HER2/Neu (Fig. 7). Moreover, $ETV1_{333-429}$ even significantly suppressed Rcl promoter activity in the absence of overexpressed HER2/Neu, consistent with its ability to act as a dominant-negative ETV1 molecule [Janknecht, 1996] that can replace any endogenous ETV1 from the Rcl promoter. In conclusion, our results demonstrate that ETV1 and HER2/Neu synergistically activate the Rcl gene promoter.

UPREGULATION OF Rcl IN BREAST CANCER

Finally, we analyzed the expression of Rcl in breast tumors. To this end, we first utilized a breast cancer array representing 50 different breast tumors and matching normal breast tissue and observed that Rcl expression was quite variable (Fig. 8A). However, when comparing the expression levels between individual tumors and their matching normal tissues, 22 out of 50 tumors showed ≥2-fold

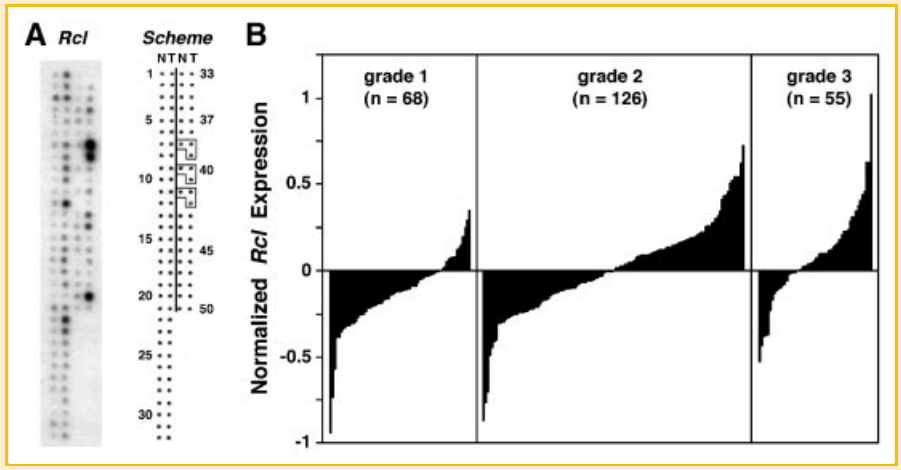

Fig. 8. Rcl overexpression in breast tumors. A: A breast cancer array representing equivalent amounts of mRNA from tumor (T) and normal (N) breast tissue was hybridized with ^{32}P-labeled human Rcl cDNA. B: Rcl expression correlates with tumor grade ($P = 3.5E-6$; Student's *t*-test). Analysis was done with ONCOMINE. Normalization was done by log 2 transforming mRNA datasets, with the median set to zero and the standard deviation set to one.

upregulation of Rcl, whereas only in one case was the amount of tumor Rcl mRNA less than half compared to the matching normal tissue. Thus, Rcl is overexpressed in ~40% of all breast tumors, an overexpression level slightly larger than the ~30% reported for HER2/Neu [Holbro et al., 2003].

Moreover, we performed in silico analysis of Rcl expression with the ONCOMINE database (www.oncomine.org). Data derived from a study that classified tumors by their Elston/Nottingham grade [Ivshina et al., 2006] demonstrate that Rcl expression rises with increasing tumor grade (Fig. 8B). Altogether, these results strongly suggest that Rcl overexpression is causally involved in breast tumorigenesis, in particular in the progression from low grade to high grade tumors.

DISCUSSION

In this report, we greatly substantiated the notion that the transcription factor ETV1 is causally involved in breast tumorigenesis by demonstrating that ETV1 is required for efficient proliferation of breast cancer cells in vitro and tumor formation in vivo. Therefore, the search for novel ETV1 target genes is highly warranted as they will allow us to deduce how ETV1 exerts its tumorigenic function in breast cells. Here, we have uncovered that Rcl is such a sought-after target gene of ETV1 based on the following observations: (i) Activation of ETV1 by HER2/Neu, or expression of the constitutively activated EWS-ETV1 and VP16-ETV1 molecules, enhanced endogenous Rcl mRNA levels. (ii) ETV1 bound to the Rcl promoter in vivo. (iii) The Rcl promoter was activated by ETV1 and HER2/Neu drastically enhanced ETV1-dependent Rcl promoter activity, whereas the dominant-negative $ETV1_{333-429}$ molecule suppressed the Rcl promoter.

Overexpression of ETV1 in itself does not lead to breast tumor formation [Netzer et al., 2002], possibly because ETV1 requires stimulation in order to become transcriptionally competent. This activation of ETV1 depends to a great extent on both phosphorylation and acetylation of ETV1, which is inducible by the HER2/Neu → Ras → Raf → MAP kinase signaling pathway [Papoutsopoulou and Janknecht, 2000; Bosc et al., 2001; Janknecht, 2001, 2003; Wu and Janknecht, 2002; Goel and Janknecht, 2003]. In particular, HER2/Neu is overexpressed in ~30% of all breast tumors [Holbro et al., 2003] and may therefore synergize with ETV1 in inducing breast cancer. In addition, the coactivator SRC-3/AIB1/ACTR is a proto-oncoprotein that is often overexpressed in primary breast tumors and causes the development of breast tumors in respective transgenic mice [Anzick et al., 1997; Torres-Arzayus et al., 2004]. Furthermore, absence of SRC-3/AIB1/ACTR inhibits HER2/Neu-mediated tumor formation in the breast, emphasizing the importance of a collaboration between HER2/Neu and SRC-3/AIB1/ACTR in carcinogenesis [Fereshteh et al., 2008]. Since ETV1 interacts with SRC-3/AIB1/ACTR [Goel and Janknecht, 2004], ETV1 may also synergize with this coactivator in inducing breast cancer. Notably, like ETV1, SRC-3/AIB1/ACTR is activated by MAP kinase phosphorylation [Font de Mora and Brown, 2000] that can be induced by HER2/Neu. Thus, ETV1, HER2/Neu and SRC-3/AIB1/ACTR may form a fateful alliance to elicit breast cancer formation involving the upregulation of Rcl gene transcription.

How could the ETV1 target gene Rcl contribute to breast tumorigenesis? On its own, Rcl has the ability to induce anchorage-independent growth in Rat1a cells, one litmus test for a potential oncogene. However, compared to a prominent oncogene such as c-Myc, Rcl's efficiency to induce anchorage-independent growth was ~10-fold lower [Lewis et al., 1997], suggesting that if Rcl is an oncogene, it would not be a potent one. In the same vein, Rcl overexpression in Rat1a cells alone did not allow them to induce tumors in athymic mice, yet Rcl synergized with lactate dehydrogenase A or VEGF to do so [Lewis et al., 2000], a further indication that Rcl is rather a tumor promoter than a classical oncogene. Notably, the VEGF gene is another target of ETV1 [Fuchs et al., 2004b], suggesting that dysregulation of ETV1 may facilitate tumorigenesis through the simultaneous activation of Rcl and VEGF gene transcription.

Recently, it was shown that the Rcl protein is a deoxynucleoside 5′-monophosphate *N*-glycosidase, converting 2-deoxynucleoside 5′-monophosphate (dNMP) into 2-deoxyribose 5-phosphate and the free base [Ghiorghi et al., 2007]. In cancer cells, the dNTP pool is elevated [Traut, 1994] which allows them to divide more rapidly. Rcl may help to keep the dNTP pool high by releasing bases from dNMPs and thus procuring bases for the nucleotide salvage pathway, explaining how upregulation of Rcl, as observed in breast tumors, may cause them to proliferate more rapidly and thereby become more aggressive. Similarly, other enzymes involved in nucleotide homeostasis such as the trifunctional protein carbamoyl-phosphate synthase/aspartate carbamoyltransferase/dihydroorotase that catalyzes rate-limiting steps of pyrimidine synthesis [Jones, 1980; Aoki and Weber, 1981] and thymidine phosphorylase that is critical for the pyrimidine nucleoside salvage pathway [Liekens et al., 2007] are upregulated in tumors and correlate with their aggressiveness.

An unsuspected function of thymidine phosphorylase was revealed when it was shown to be identical to platelet-derived endothelial cell growth factor. Thymidine phosphorylase catalyzes the reaction thymidine + phosphate ↔ thymine + 2-deoxyribose 1-phosphate, and the latter can be dephosphorylated to 2-deoxyribose that acts as an endothelial cell chemoattractant and angiogenic factor [Brown and Bicknell, 1998; Akiyama et al., 2004]. Likewise, Rcl's enzymatic action leads to increased levels of 2-deoxyribose 5-phosphate that can be dephosphorylated to 2-deoxyribose in the cell. Thus, Rcl may foster 2-deoxyribose-dependent angiogenesis and thereby contribute to tumorigenesis.

In conclusion, our results point out a mechanism of how Rcl upregulation in breast tumors is brought about by ETV1 in collaboration with HER2/Neu and/or SRC-3/ACTR/AIB1. Moreover, the fact that upregulation of Rcl correlates with increased tumor aggressiveness strongly suggests a causal role of Rcl in breast tumorigenesis. To definitely prove this, transgenic mouse models of Rcl should be established to investigate if Rcl overexpression on its own leads to tumorigenesis, or if it supports tumorigenesis induced by established oncogenes and carcinogens. If so, Rcl will be a promising new drug target, since it is an enzyme for which small molecule inhibitors can be developed that are systemically applicable and may delay tumor vascularization and cell proliferation.

ACKNOWLEDGMENTS

We thank the team led by Cathy Chang and Brian Hilbush at Digital Gene Technologies for performing the TOGA procedure, Saskia Brandt and Kari Rossow for assistance with immunofluorescence microscopy, and Bruno Fuchs for help in constructing Rcl expression vectors. We gratefully acknowledge financial support by the Department of Defense Breast Cancer Research Program (DAMD17-03-1-0538), the Fraternal Order of Eagles Cancer Research Fund, the Breast Cancer Research Foundation, and the Bruce and Martha Atwater Foundation.

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