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Overexpression of RasN17 Fails to Neutralize Endogenous Ras in MCF7 Breast Cancer Cells

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Receptor tyrosine kinases of the ErbB family have been implicated in the onset/progression of a number of neoplasias. In these diseases, ErbB receptor expression may be accompanied by constitutive activation caused by molecular alterations, overexpression, or ligand binding. An important signaling route activated by these receptors that has been linked to the stimulation of cell proliferation is the Ras route. Here we have investigated the action of a mutant Ras form, H-RasN17, on the proliferation of the breast cancer epithelial cell line MCF7 cells. In these cells expression of RasN17 failed to affect serum or ErbB receptor–stimulated proliferation. Analysis of the action of RasN17 indicated that overexpression of this mutant form of Ras did not affect neuregulin or protein kinase C–induced activation of Erk1/2. In addition, RasN17 failed to prevent activation of endogenous N-Ras and H-Ras even though the levels of the latter were much lower than those of the RasN17 form. The failure of RasN17 to prevent endogenous Ras activation did not appear to be due to deficient processing or sorting of the mutated form. These data indicated that the action of RasN17 as a *bona fide* inhibitor of Ras depends on the cell type and requires detailed analysis of the biochemical and biological properties of RasN17, particularly with respect to the activation of endogenous Ras.

Key words: ErbB, MAPK, neuregulin, Ras.

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HB-EGF, heparin binding EGF–like growth factor; Erk, extracellular signal–regulated kinase; GEF, guanine nucleotide exchange factor; PMA, phorbol 12-myristate, 13-acetate; PKC, protein kinase C; BIM, bisindolylmaleimide; PI3K, phosphatidylinositol 3-kinase; TGFα, transforming growth factor α; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PBST, PBS supplemented with 0.1% Triton X-100.

The ErbB family of receptor tyrosine kinases has been shown to play important roles in several physiological and pathological processes (*1*). These receptors are essential for proper development, and absence of each of these receptors has been shown to result in malformation of several organs, including the heart (*2*, *3*) and the nervous system (*4*), that causes embryonic lethality. In addition, overexpression of these receptors, particularly ErbB2 and the EGFR, has been associated with several types of solid tumors, including breast, ovarian, lung, head and neck, and nervous system neoplasias (*5*).

In mammals, four related ErbB receptors have been described: ErbB1/EGFR, ErbB2/neu, ErbB3, and ErbB4 (*6*). Activation of these receptors occurs by oligomerization, normally driven by the interaction with their ligands (*6–8*). A large family of over 20 ligands, collectively included in the EGF family, is responsible for the activation of these receptors (*9*). With respect to their interaction with the different ErbB receptors, these ligands may be clustered into three types (*6*, *10*): those that interact with the EGFR, such as EGF or proTGFα; those that interact with ErbB3 and ErbB4, such as the neuregulins (NRG), and those that may interact with the EGFR and ErbB3/ErbB4, such as betacellulin or HB-EGF. Under physiological conditions in which ErbB2 expression is limited, activation of ErbB2 occurs by heterooligomerization with other receptors of the ErbB family (*11*). However, when ErbB2 is overexpressed, as occurs in certain types of neoplasias, the likelihood of ErbB2–ErbB2 interactions increases and spontaneous oligomerization results in ErbB2 activation in the absence of ligand (*12*).

Activation of the intrinsic tyrosine kinase activity of these receptors results in the phosphorylation of the receptors on specific tyrosine residues (*13*). This is followed by the interaction of signaling molecules with tyrosine-phosphorylated residues located in the endodomain of these receptors (*14*). This phenomenon may be followed by phosphorylation and activation of these signaling molecules, or their recruitment to the plasma membrane where they may interact with and activate other signalling mediators.

An important signaling route activated by receptor tyrosine kinases of the ErbB family is the Ras pathway (*15*). Activation of this route occurs by transition of Ras from Ras-GDP to Ras-GTP, a reaction favoured by the Ras guanine nucleotide exchange factors (GEFs). Tyrosine phosphorylation of receptor tyrosine kinases results in the recruitment of GEFs to the plasma membrane. This brings inactive Ras into proximity with the GEFs that increase the dissociation rate of GDP allowing GTP

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to bind to Ras. Activated Ras is then responsible for the activation of signaling cascades such as the Raf/Erk or the PI3K/Akt route (*15*, *16*). Activation of the mitogen-activated protein kinases Erk1 and Erk2 MAPK cascades has been shown to contribute to the proliferation of breast cancer cells in response to ErbB receptor activation. Thus, addition of NRG has been shown to stimulate Erk1/2 dual phosphorylation, and treatment with agents that prevent Erk1/2 activation has been reported to inhibit proliferation of breast cancer cells in response to NRG (*17*, *18*).

Studies on the role of Ras in cell proliferation have mainly employed a mutant form of Ras (RasN17) in which serine at position 17 is replaced by asparagine (*15*). This mutant form has been shown to act by sequestering Ras-GEF, and transfection into NIH3T3 rodent fibroblasts causes cessation of cell proliferation (*19*). However, in the breast epithelial cell line MCF10A expression of RasN17 has been reported to prevent EGF-induced activation of Erk1/2 without significantly affecting cell proliferation (*20*). In these cells, however, EGF-induced cell proliferation was sensitive to the expression of a dominant negative form of the big MAPK Erk5, which in these cells was activated by EGF even in the presence of RasN17. Therefore, the possibility exists that in breast epithelial cells the control of cellular proliferation by receptor tyrosine kinases of the ErbB family could occur by Ras-independent routes. Here we have investigated the action of RasN17 on NRG-induced proliferation of MCF7 breast epithelial cancer cells. In contrast to the marked inhibitory effect on NIH3T3 cell proliferation, we found that overexpression of RasN17 failed to prevent serum and NRG-induced proliferation. Overexpressed RasN17 was correctly processed and targeted to the plasma membrane, but failed to inhibit endogenous N-Ras, H-Ras and Erk1/2 activation in response to NRG. These results suggest that the actions of RasN17 are cell-type specific, and indicate that breast epithelial–derived cancer cells may be resistant to the action of this dominant negative form of Ras.

MATERIALS AND METHODS

Reagents and Immunochemicals—Cell culture media, sera and G418 were purchased from Gibco Brl (Gaithersburg, MD). Doxycycline, MTT, PMA, luminol and 4-*p*-iodophenol were from Sigma Chemical Co. Hygromycin B, and the PKC inhibitor BIM were from Calbiochem. Immobilon P membranes were from Millipore Corp. (Bedford, MA). Other generic chemicals were purchased from Amersham-Pharmacia, Roche Biochemicals, or Merck.

The anti–pan Ras monoclonal antiserum M-90 was generously provided by Dr. Eugenio Santos (Centro de Investigación del Cáncer, Salamanca, Spain). The rabbit polyclonal anti–H-Ras, anti–N-Ras, anti-Erk2, and the mouse monoclonal antiphosphotyrosine, anti–phospho-Erk, and anti–K-Ras antibodies were from Santa Cruz Biotechnology. The mouse monoclonal anti–phosphoP-KBα/Akt antibody was from Transduction Laboratories. The monoclonal anti–ErbB2 ectodomain antibody 4D5 and the recombinant soluble NRG were provided by Dr. Mark. X. Sliwkowski (Genentech, San Francisco, CA). The Ab3 anti-ErbB2 antibody used for Western blotting was from Oncogene Science. The anti-ErbB3 and anti-ErbB4 antibodies were from Dr. Matthias H. Kraus (Istituto Europeo di Oncologia, Milan, Italy). HRP conjugates of anti-rabbit IgG and anti-mouse IgG were from BioRad Laboratories (Cambridge, MA). The anti–mouse Cy2–conjugated secondary antibody was from Jackson Immunoresearch.

Cell Culture, Transfections, and Retrovirus Production—All cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2–95% air. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing a high glucose concentration (4,500 mg/liter) and antibiotics (penicillin at 100 U/ml, streptomycin at 100 μg/ml) and supplemented with 10% fetal bovine serum (MCF7, NIH3T3 and 293T cells).

For transient production of retroviruses, 293T cells were plated in 60-mm-diameter dishes (1.8×10^6 cells in 3 ml of DMEM with 10% FBS) and allowed to attach overnight. Five minutes prior to transfection, 25 μM chloroquine was added to each plate. The transfection solution contained DNA [2.5 μg of pMD-G, 5 μg of pNGVL-MLV-gag-pol, 3 μg of retroviral vector (pLZR-RasN17-IRES-GFP, or pLZR-IRES-GFP alone)], 61 μl of 2 M $CaCl_2$, and double-distilled H_2O to 500 μl. After mixing, 0.5 ml of 2× HBS (pH 7.0) was added and the solution was bubbled for 15 s. The HBS-DNA complex was then dropped on the cells. Eight hours later, the medium was replaced with fresh complete culture medium that 24 to 32 h posttransfection was again replaced with 3 ml of fresh virus-collecting medium. Twenty-four hours after the medium change, the supernatant from transfected cells was collected and centrifuged at $1,000 \times g$ for 5 min. Twenty-four hours before infection, MCF7 or NIH3T3 cells were plated at 20,000 cells per well in 24-well plates or 200,000 cells per 60-mm plate, and infected with viral supernatants containing Polybrene at 6 μg/ml. The following day, the medium was replaced with normal culture medium containing 10 nM NRG were indicated. Cell proliferation was analyzed by an MTT-based assay, and cells were analyzed for infected protein expression by Western blotting with the anti–H-Ras antibody as indicated below.

To generate MCF7 cells that expressed RasN17 in a regulated manner, we used the tetracycline transactivator system. MCF7-TetOff cells [obtained either from R. Michalides (*21*) or from Clontech] were transfected with pTRE2-RasN17, or with pTRE2 (Clontech) together with pBabe-Puro using Lipofectamine (Gibco-BRL). Clones were then cultured in normal medium supplemented with 10 ng/ml doxycycline and selected with puromycin at 3 μg/ml. The expression of H-RasN17 was analyzed by Western blotting comparing the amounts of RasN17 in the absence and presence of doxycycline at 10 ng/ml.

Construction of Plasmids and Fusion Proteins—To generate inducible clones of MCF7 cells, the cDNA coding for RasN17 (obtained from Dr. P. Crespo, Instituto de Investigaciones Biomédicas, Madrid) was subcloned into the *Bam*HI/*Not*I sites of the pTRE2 vector or into the *Bam*HI/*Xho*I sites of pLZR-IRES-GFP. All the plasmids were sequenced to verify the Ser17 to Asn17 mutation. The plasmids for the generation of the fusion proteins GST-Raf^{RBD} and GST-RalGDS^{RBD}, were from Drs. Piero

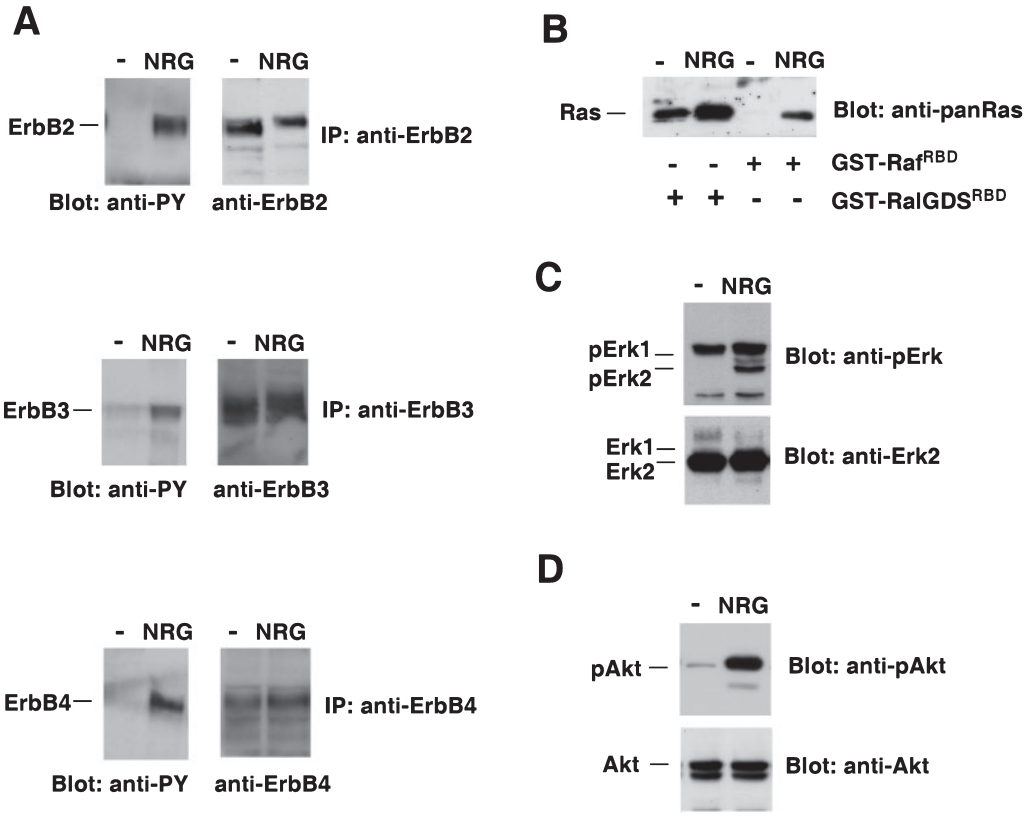

Fig. 1. **A: Effect of NRG on ErbB receptor activation in MCF7 cells.** Cells were plated in 60-mm dishes and treated with NRG (10 nM) for 15 min where indicated. Cell extracts were prepared and immunoprecipitated with antibodies specific for each of the ErbB receptors analyzed. Blots were probed with anti-PY or anti-ErbB receptor antibodies as indicated. **B: Effect of NRG on the association of Ras with GST-Raf^{RBD} or GST-RalGDS^{RBD}.** Cells were stimulated with NRG as above and lysates were precipitated with the indicated GST fusion proteins as described under "MATERIALS AND METHODS." Precipitates were run on 12% SDS-PAGE gels and blots were probed with anti-panRas antibodies. **C and D: Activation of Erk1/2 and Akt by NRG.** MFC7 cells were treated with NRG as above. Blots of cell lysates were probed with anti-pErk1/2 or Erk2 antibodies (C); or anti-pAkt or Akt antibodies (D).

Crespo and E. Santos. Plasmids were transformed in *E. coli* strain DH5α, and the bacteria were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C. Bacteria were lysed, and the fusion proteins were isolated from the lysate by glutathione-Sepharose affinity chromatography. The fusion proteins were eluted from the resin with reduced glutathione, and the amount of protein was quantitated by SDS-PAGE using bovine serum albumin (BSA) as standard.

Immunoprecipitation and Western Blotting—Cells were washed with phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer [140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 20 mM Tris (pH 8.0), 1 μM pepstatin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate]. Cells were scraped from the dishes, and lysates were centrifuged at 10,000 × *g* at 4°C for 10 min. Supernatants were then transferred to new tubes with the corresponding antibody and Protein A-Sepharose. Immunoprecipitations were performed at 4°C for at least 2 h, and the immune complexes were recovered by a short centrifugation, followed by three washes with 1 ml of cold lysis buffer. Samples were then boiled in electrophoresis sample buffer and loaded in SDS-PAGE gels. After transfer to PVDF membranes, filters were blocked for 1 h in TBST containing 1% BSA and then incubated for 2 to 16 h with the corresponding antibody. Filters were washed three times with TBST and then incubated with horseradish peroxidase–conjugated secondary antibodies for 30 min, and bands were visualized by a luminol-based detection system with *p*-iodophenol enhancement (*22*).

Cell Proliferation Measurements—Cell proliferation was analyzed by an MTT-based assay as described (*23*). Cells were plated in 24-well plates to a density of 20,000 per well. Cultures were allowed to attach overnight before treatment with different concentrations of serum in the presence or absence of NRG (10 nM) and doxycycline (10 ng/ml). In parallel, 24 h after the cells were plated, an MTT assay was performed and considered the starting point before treatments. The medium in each well was replaced with 250 μl of fresh medium containing MTT at 0.5 μg/ml and plates were returned to the incubator for 1 h. The medium-MTT was then removed, 500 μl of dimethyl sulfoxide was added to each well, and the plate was kept in agitation for 5 min in the dark to dissolve the MTT-formazan crystals. The absorbance of the samples was then recorded at 570 nm. Three wells were analyzed for each condition, and wells containing medium plus MTT but no cells were used as blanks. Unless otherwise indicated the results are presented as the mean ± the standard deviation (SD) of triplicates of a representative experiment that was repeated at least three times. In parallel, wells of cells being treated or not with doxycycline were lysed at the different times at which the MTT assays were performed, and RasN17 content was analyzed by Western blotting.

Ras Binding to GST-RalGDS^{RBD} or GST-Raf^{RBD}—Cells were stimulated for 15 min with 10 nM NRG and lysed with ice-cold lysis buffer containing 25 mM HEPES pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1 mM sodium orthovanadate, 25 mM NaF, 1 mM PMSF, 1 μM pepstatin, 1 μg/ml aprotinin,

and 1 μg/ml leupeptin. After centrifugation at 12,000 × *g* for 10 min, cellular extracts were precipitated with 15 μg of GST-RalGDS^{RBD} or GST-Raf^{RBD} for 30 min at 4°C. Samples were then washed three times with lysis buffer, resuspended in 2× sample buffer, and boiled for 5 min. The samples were run in 12% gels and Ras bound to the fusion protein analyzed by Western blotting with the M90 or isoform-specific anti-Ras antibodies.

Confocal Immunofluorescence Microscopy—Cells plated on coverslips were washed with PBS and fixed in 2% *p*-formaldehyde for 30 min at room temperature. Monolayers were washed twice in PBST (PBS supplemented with 0.1% Triton X-100 final) and then blocked in PBST with 5% BSA for 1 h at room temperature. Monolayers were then incubated with the primary antibody in blocking solution for 2 h at room temperature. After two washes of 15 min each in PBST, the coverslips were incubated with Cy2-conjugated secondary antibodies for 30 min, washed three times for 5 min each time in PBST, and mounted. Samples were analyzed by confocal immunofluorescence microscopy using a Zeiss LSM 510 confocal microscope.

RESULTS

Addition of NRG to MCF7 cells caused tyrosine phosphorylation of the ErbB2, ErbB3 and ErbB4 receptor tyrosine kinases (Fig. 1A), but had little effect on EGFR tyrosine phosphorylations, probably due to the low level of EGFR expression (see Ref. *23*, and data not shown). To analyze the action of NRG on Ras in these cells, we used an assay based on the ability of activated Ras to interact with the Ras binding domain (RBD) of Raf or RalGDS (*24*). Under resting conditions, and after 24 h of serum deprivation, preincubation of cell lysates from MCF7 cells with GST-RalGDS^{RBD} resulted in the precipitation of a significant amount of Ras, identified by Western blotting with an anti-panRas antibody (Fig. 1B). Addition of NRG increased the amount of Ras bound to GST-RalGDS^{RBD}. The latter effect of NRG was also evidenced when using GST-Raf^{RBD} to pull down activated Ras. However, GST-Raf^{RBD} was less efficient than GST-RalGDS^{RBD} in its interaction with Ras under resting conditions, suggesting that most of the activated Ras present under resting conditions may preferentially be bound to RalGDS in these cells. NRG addition also caused stimulation of the phosphorylation of Erk1/2 (Fig. 1C) and Akt (Fig. 1D), two signaling pathways that can be activated by the Ras route (*15*).

To analyze the role of Ras in NRG-induced cell proliferation, a form of H-Ras (RasN17) known to act as a dominant negative was used (*19*). Because of the poor transfection efficiencies of MCF7 cells, we used a retroviral system to express RasN17 in these cells. The cDNA coding for the mutant protein was subcloned into the pLZR-IRES-GFP retroviral vector and sequenced to verify the Ser17 to Asn17 mutation in the final retroviral construct. Viral supernatants obtained from 293T cells were then used to infect NIH3T3 or MCF7 cells. Western blotting of cell lysates using an H-Ras specific antibody demonstrated that RasN17 expression was several times higher than normal MCF7 or NIH3T3 H-Ras levels (Fig. 2A, and data not shown). In agreement with a previous report (*19*), expression of RasN17 in NIH3T3 cells

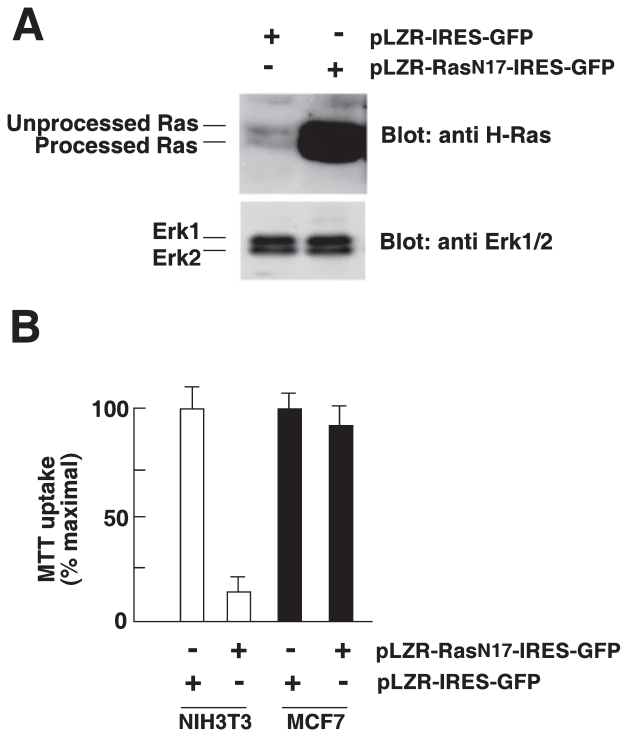

Fig. 2. **Action of RasN17 on MCF7 proliferation.** A: Expression of RasN17 by retroviral infection. MCF7 cells were infected with a retrovirus coding for RasN17 (pLZR-RasN17-IRES-GFP), or with viruses containing only the vector (pLZR-IRES-GFP). Three days after the infection, cell lysates were prepared and analyzed for expression of RasN17 by Western blotting with anti–H-Ras antibodies. Analysis of the amount of Erk1/2 by Western blotting (bottom panel) was used as a loading control. B: Proliferation of NIH3T3 and MCF7 cells infected with pLZR-IRES-GFP or pLZR-RasN17-IRES-GFP. Infected cells were plated in 24-well dishes and cultured for 4 days. MTT was then added and cultures incubated for an additional hour in the presence of the dye. Data are presented as percentage of growth of cells infected with the empty vector (mean ± SD of triplicates).

profoundly inhibited cell growth (Fig. 2B). However, in epithelial MCF7 cells expression of RasN17 at levels analogous to those that caused profound growth inhibition of NIH3T3 cells did not substantially affect proliferation of MCF7 cells.

We reasoned that if RasN17 would fail to prevent proliferation of MCF7 cells, it would thus be possible to generate clones of MCF7 cells stably overexpressing RasN17. In line with this hypothesis, G418 selection of MCF7 cells resulted in the isolation of a substantial number of clones expressing RasN17 (Fig. 3A). Parallel transfections of RasN17 into NIH3T3 cells resulted in the isolation of very few (five) G418-resistant clones, none of them expressing the RasN17 mutant protein (data not shown).

RasN17 appeared as two bands, a faster migrating band that represented processed Ras, and a slower migrating band corresponding to unprocessed Ras (*25*). In the isolated clones, most of the exogenously expressed RasN17 appeared as the processed form, independently of the expression level of RasN17. When proliferation of two clones expressing the RasN17 form (clones 19 and 25) was compared to that of control clones (clones 2 and

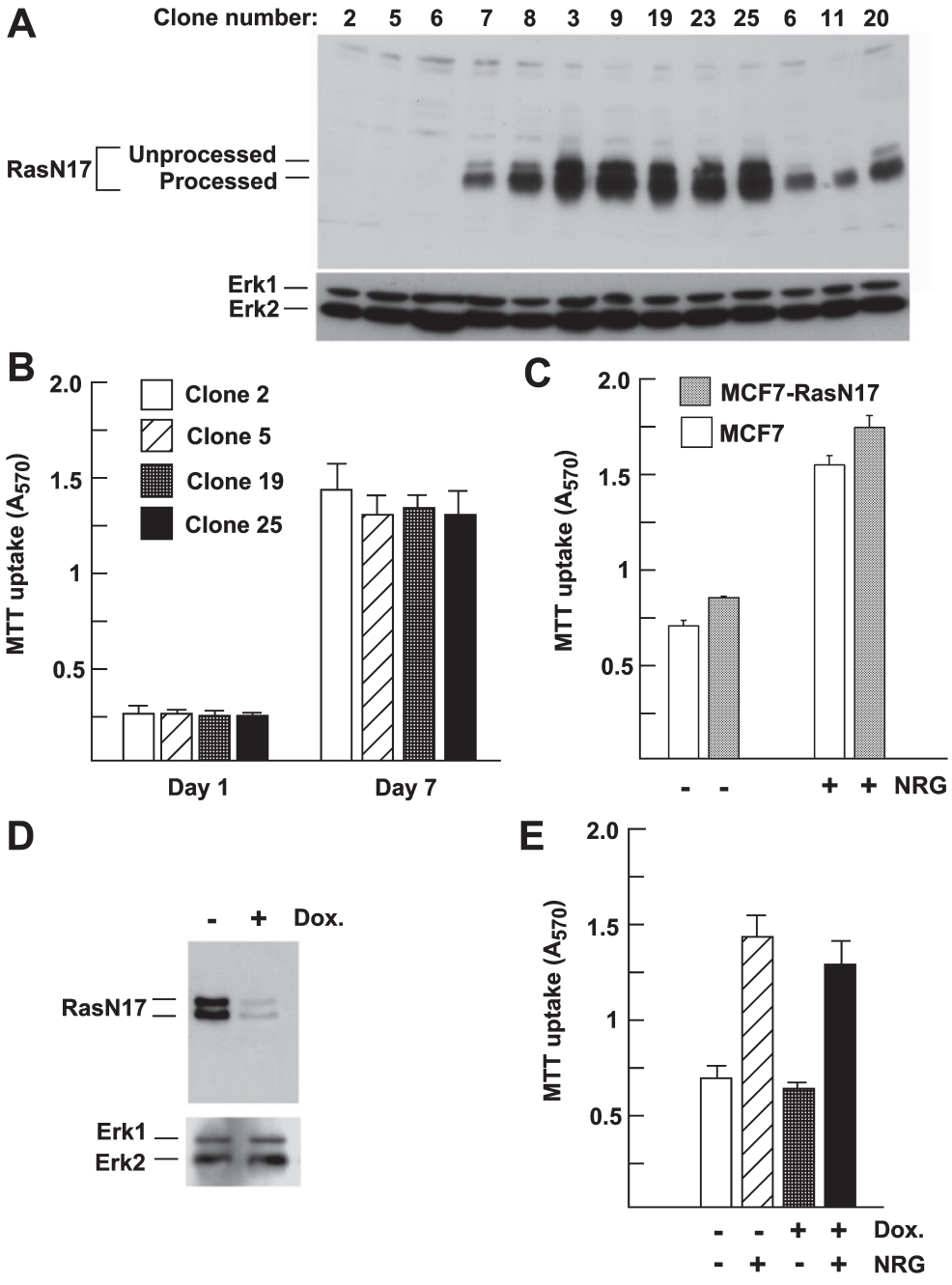

Fig. 3. **A: Expression of RasN17 in several MCF7 clones.** MCF7 cells were transfected with pCDNA3-RasN17 and selected by treatment with G418. Clones resistant to the antibiotic were isolated, and their expression was analyzed by Western blotting with the anti–H-Ras antibody. As a loading control (bottom blot), we performed Western blotting with anti-Erk1/2 on equal amounts of cell lysates from the distinct cell lines. **B: Proliferation of several clones of MCF7 cells transfected with RasN17.** Cells were plated in 24-well dishes in the presence of complete media containing 10% serum, and MTT absorbance was measured at the times indicated. **C: The action of NRG (10 nM) on MCF7 and MCF7-RasN17$^{clone\ 19}$ is shown. D: Regulated expression of RasN17 in MCF7-TetOff cells.** Cells were maintained in the absence or presence of 10 ng/ml of doxycycline and then analyzed by Western blotting for the presence of RasN17. An anti-Erk1/2 Western blot (bottom panel) was used as a loading control. E: The panel shows the effect of NRG (10 nM) on the proliferation of the MCF7-RasN17^{TetOff} clone in the presence or absence of doxycycline.

5) that did not express RasN17, no substantial difference was observed in their growth rates at serum concentrations ranging from 0.1–10% (Fig. 3B, and data not shown).

The proliferation in response to NRG was next analyzed. To this end, MCF7 and MCF7-RasN17$^{clone\ 19}$ cells were plated in 24-well dishes and allowed to attach overnight in medium containing serum. The medium was then replaced with serum-free medium, and proliferation was measured three days later. In the presence of NRG (10 nM) the proliferation rates of MCF7 and MCF7-RasN17 cells were analogous (Fig. 3C).

To further explore this point and to avoid any possible clonal differences in proliferation behaviour, we made use of an expression system that allowed regulated expression of RasN17. The cDNA coding for RasN17 was inserted into the pTRE2 vector, which allows for efficient expression of proteins under the control of the tetracycline transactivator, and the cDNA was transfected into MCF7-TetOff cells. Clones were selected with hygromycin in the presence of 1 μg/ml of doxycyclin to down-regulate the expression of RasN17. Upon selection of stable clones, RasN17 expression was analyzed upon release of inhibition by removal of doxycyclin. As shown in Fig. 3D, expression of RasN17 was substantially up-regulated when doxycyclin was removed from the culture media. As occurred with stable cell lines, NRG stimulated proliferation of MCF7-RasN17^{TetOff} cells independently of the expression of RasN17 (Fig. 3E).

The failure of RasN17 to affect MCF7 cell proliferation led us to investigate the action of this mutant on the Ras/

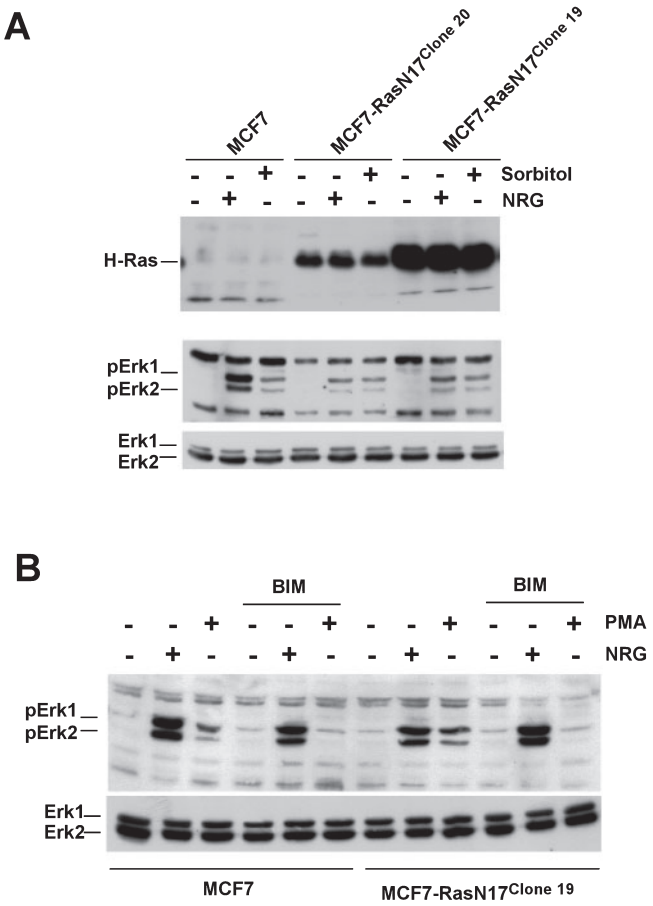

Fig. 4. **Effect of RasN17 on the activation of Erk1/2 through different intracellular pathways.** A: MCF7 and two clones expressing different amounts of RasN17 were treated with NRG or sorbitol, and the activation of Erk1/2 was analyzed as described above by the use of phosphospecific anti-pErk1/2 antibodies. As a control of loading we used an anti-Erk1/2 Western blot (bottom panel). B: Action of the PKC inhibitor BIM on NRG and PMA-induced Erk1/2 activation. Cells were preincubated for 15 minutes with BIM (10 μM) before PMA (1 μM) or NRG addition where indicated. Blots were probed with anti-pErk (top panel) or anti-Erk (bottom panel) antibodies.

MAPK signal transduction pathway. First, we evaluated the action of RasN17 on NRG and osmotic stress-induced Erk1/2 activation in the wild type and two clones of MCF7 cells expressing different amounts of RasN17 (Fig. 4A, top panel). NRG or sorbitol caused dual phosphorylation of Erk1/2 as identified by Western blotting with the anti-pErk1/2 antibody (Fig. 4A, bottom panel). The effect of these treatments on Erk1/2 activation did not substantially change in the two RasN17-expressing clones.

Since PKC-induced Erk1/2 activation has been reported to be unaffected by RasN17 (*26*), the possibility that NRG-induced Erk1/2 activation occurred by a PKC-mediated route was evaluated. Treatment of MCF7 cells with PMA stimulated Erk1/2 dual phosphorylation, and this action of the phorbol ester was largely inhibited by preincubation with the PKC inhibitor bisindolylmaleimide (BIM) (Fig. 4B). Analogously, in MCF7-RasN17 cells PMA-induced Erk1/2 activation was unaffected by the expression of RasN17, but was also inhibited by BIM. The action of NRG on Erk1/2 activation was preserved in wild-type and RasN17-expressing MCF7 cells treated with the PKC inhibitor (Fig. 4B), indicating that NRG-induced Erk1/2 activation may occur through PKC-independent routes.

We next analyzed to what extent RasN17 could affect endogenous Ras activation. To this end, we treated MCF7-RasN17^{TetOff} cells with NRG and analyzed Ras bound to GST-Raf^{RBD}. As shown in Fig. 5A, treatment of these cells with NRG caused a significant increase in GST-Raf^{RBD}-bound Ras, and this association was unaffected by the expression of RasN17. Analysis of Ras isoforms in MCF7 cells with antibodies that specifically recognize individual isoforms indicated that N-Ras activation by NRG was largely unaffected by the expression of RasN17 (Fig. 5B). This finding was also confirmed by using MCF7-RasN17$^{clone\ 19}$ cells, which expressed a much higher amount of RasN17 (Fig. 5C). Expression of endogenous H-Ras in cell extracts from MCF7 cells was lower than the expression of N-Ras, and the pull-down experiments indicated that the presence of RasN17 did not prevent NRG-induced H-Ras activation (Fig. 5B, and data not shown). Expression of K-Ras was very low, and for this reason the action of RasN17 on its activation was difficult to evaluate (Fig. 5B and data not shown).

To analyze whether the failure of RasN17 to prevent endogenous Ras activation was due to missorting of the dominant negative form we performed immunofluorescence analysis of RasN17 distribution with the anti–H-Ras specific antibody. Confocal imaging of wild-type MCF7 cells indicated very low expression of endogenous H-Ras (Fig. 5D), in agreement with the Western blotting data (see above). In MCF7RasN17^{TetOff} cells the mutant protein was easily detected and mainly targeted to the plasma membrane (Fig. 5D).

DISCUSSION

The Ras proteins have been considered important regulators of cellular proliferation (*27*, *28*). Mutations in these proteins are frequent in a number of malignancies, especially in those derived from the pancreas (*28*). However, in the breast the frequency of Ras mutations has been reported to be low (*28*). While this low appearance excludes Ras as a critical oncogene in breast cancer, signaling routes in which Ras participates, such as the Erk1/2 and the PI3K/Akt signaling pathways, have been implicated in ErbB receptor-mediated proliferation and survival of breast cancer cells. In fact, in T47D cells, inhibition of the Erk1/2 route by the MEK inhibitor PD98059 blocked G1/S transition induced by NRG (*29*). In addition, treatment with LY294002, an inhibitor of the PI3K/Akt route, also prevented NRG-induced cell cycle progression in ErbB2-overexpressing SKBR3 cells (*17*).

Here we intended to investigate the potential role of Ras in NRG signal transduction in the breast cancer cell line MCF7. Much of the knowledge accumulated on the role of Ras in signal transduction and proliferation has been gained by the use of dominant negative forms of Ras (*15*). These mutant forms of Ras, particularly the H-RasN17 form, act by sequestering Ras GEF function and have been shown to inhibit the proliferation of a number

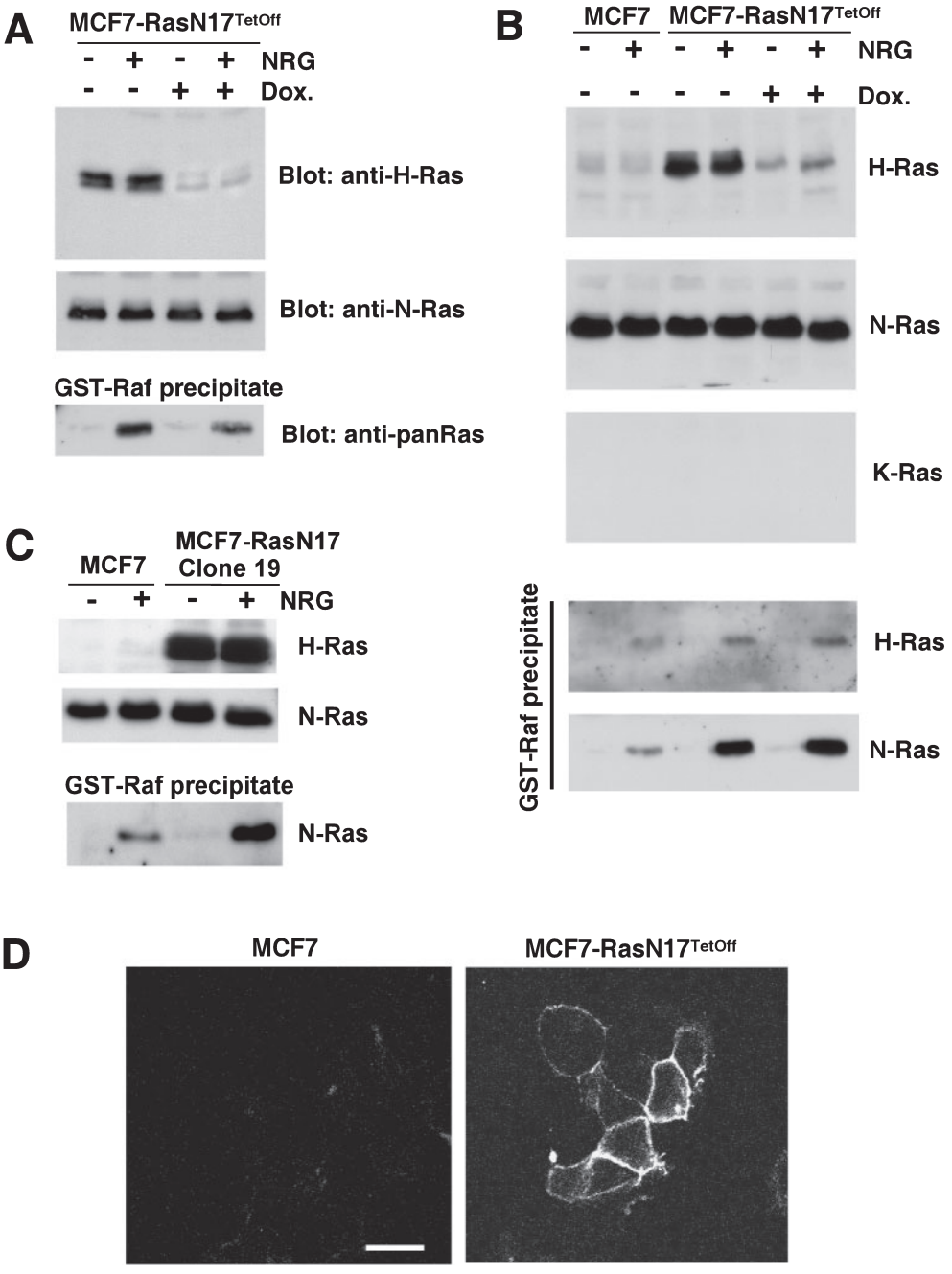

Fig. 5. **Effect of RasN17 on NRG-induced Ras activation.** A: Action of NRG on the association of Ras with GST-Raf^{RBD} in MCF7-RasN17^{TetOff} cells. Cells maintained in the absence or presence of doxycycline were stimulated with NRG (10 nM, 15 min), and lysates were precipitated with the GST-Raf^{RBD} fusion protein as described under "MATERIALS AND METHODS." Precipitates were run on 12% SDS-PAGE gels, and blots were probed with anti-panRas antibodies (bottom panel). The effect of doxycycline on the amount of the different Ras isoforms was analyzed by Western blotting using specific anti-H, or anti-N antibodies. B: Effect of RasN17 on the activation of the different Ras isoforms. The experiment was performed as described in A, but the GST-Raf^{RBD} precipitates were probed with specific anti–H-Ras or anti–N-Ras antibodies. C: Action of RasN17 on N-Ras activation in MCF7-RasN17$^{clone\ 19}$ cells. Conditions for NRG treatment and measurement of N-Ras were as in A. D: Immunofluorescence analysis of H-Ras expression in MCF7 and MCF7-RasN17^{TetOff} cells. Cells were plated on glass coverslips, and H-Ras expression was analyzed by the use of the anti–H-Ras antibody C-20. Bar = 30 μM.

of cell lines, the prototypic model being NIH3T3 rodent fibroblasts (*19*). However, the fact that most Ras mutations in humans arise from epithelial-derived cells raises the important question of whether the data so far obtained in rodent mesenchymal–derived fibroblasts can be extrapolated to epithelial-derived tissues. Interestingly, in non-tumoral breast epithelial cell lines such as MCF10A expression of the RasN17 mutant has been shown to inhibit EGF-induced Erk1/2 activation without affecting growth factor-induced proliferation (*20*). Therefore, it is likely that Ras participates in these cells in EGFR-mediated Erk1/2 activation, but may not play a major role in EGFR-mediated stimulation of cell growth (*20*).

Expression of RasN17 in MCF7 cells failed to prevent serum or NRG-induced proliferation. This was observed using different experimental approaches to overexpress RasN17, including retroviral transduction, regulated expression, and clones isolated upon G418 selection. The latter is in profound contrast to the results obtained using NIH3T3 fibroblasts. Attempts to generate clones of NIH3T3 fibroblasts constitutively expressing RasN17 failed. The only G418-resistant clones isolated failed to express RasN17 when examined for H-Ras expression by Western blotting using H-Ras specific antibodies. In addition, in cell proliferation experiments in which we used retroviral infection, NIH3T3 proliferation was strongly compromised in cells infected with RasN17, in

contrast to the lack of a significant effect of RasN17 on MCF7 proliferation. Apparently this lack of effect of RasN17 on MCF7 breast cancer cells cannot be extrapolated to every epithelial-derived tissue. Thus, expression of RasN17 in the epidermis has been shown to reduce Ras activation and cause differentiation of epidermal progenitor cells (*30*).

As with cell proliferation responses, NRG-induced activation of Erk1/2 appeared to be largely insensitive to the presence of RasN17 in MCF7 cells. Although in the case of other ErbB receptors activation of Erk1/2 has been reported to occur in a Ras-independent manner (*20*), a role of Ras in NRG-induced Erk1/2 activation in MCF7 cells cannot be fully excluded. In fact, the failure of RasN17 to prevent Erk1/2 activation by NRG, sorbitol, or the PKC activator PMA may be due to the inability of RasN17 to prevent endogenous Ras activation in MCF7 cells. This is indicated by the comparative analysis of Ras activation in MCF7 and MCF7^{RasN17} TetOff cells. Overexpression of RasN17 to levels several times higher than that of endogenous H-Ras did not inhibit binding of H-Ras to GST-Raf^{RBD}. Mechanistically, the reason for this failure does not appear to be a deficient maturation or targeting of RasN17. In fact, Western blot analysis indicated that RasN17 was processed in MCF7 cells, and immunofluorescence experiments demonstrated that a significant proportion of RasN17 was targeted to the plasma membrane.

In addition to the effect on RasN17 on NRG-induced Erk1/2 activation, the mutant also failed to prevent PMA or sorbitol-induced Erk1/2 activation. This is in line with previous observations that PKC-mediated activation of Erk1/2 is not substantially affected by the RasN17 mutant (*26*, *31*). This may be due to the fact that activation of Erk1/2 in response to PMA is Ras-independent, as also reported for other cellular activators (*32*). Alternatively, preexisting latent signaling complexes of Ras-GTP with components of the Erk route could also explain the resistance of Erk1/2 activation by different stimulators to the presence of RasN17. This hypothesis is based on the PKCε-Raf-N-Ras complexes previously shown to exist in other cells lines, and that also appear to be insensitive to the dominant-inhibitory effect of RasN17 (*33*). In this respect it is interesting to note that GST-RalGDS^{RBD} was able to pull down Ras in serum-starved MCF7 cells. If this Ras isoform were N-Ras (as this was the major isoform expressed in MCF7 cells) in a GTP-bound state, then the possibility of N-Ras-GTP constitutes a latent signaling module could fit with a model in which receptor tyrosine kinase–induced Ras activation could be resistant to inhibition by overexpression of RasN17.

Whatever the mechanism by which MCF7 cells escape to the action of RasN17, the results herewith presented indicate that analysis of the action of this mutant form of Ras as a *bona fide* inhibitor requires careful evaluation of the action RasN17 on endogenous Ras isoforms, and on the signaling pathways under the potential control of Ras.

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