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ARTICLES

Enhanced Expression of Heregulin in c-*erb* B-2 and c-Ha-*ras* Transformed Mouse and Human Mammary Epithelial Cells

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Abstract Heregulin β1 was found to stimulate the anchorage-dependent, serum-free growth of nontransformed human MCF-10A mammary epithelial cells. Unlike epidermal growth factor, transforming growth factor α, or amphiregulin, heregulin β1 was also able to induce the anchorage-independent growth of MCF-10A cells. In contrast, the anchorage-dependent, serum-free growth of c-Ha-*ras* or c-*erb* B-2 transformed MCF-10A cells was unaffected by heregulin β1, whereas heregulin β1 was able to stimulate the anchorage-independent growth of these cells. c-Ha-*ras* or c-*erb* B-2 (c-*neu*) transformed MCF-10A or mouse NOG-8 mammary epithelial cells express elevated levels of 2.5, 5.0, 6.5, 6.8, and 8.5 kb heregulin mRNA transcripts and/or synthesize cell-associated 25, 29, 50, and 115 kDa isoforms of heregulin. Since the MCF-10A cells and transformants also express c-*erb* B-3, these data suggest that endogenous heregulin might function as an autocrine growth factor for Ha-*ras* or *erb* B-2 transformed mammary epithelial cells.

Key words: heregulin, transformation, *erb* B-2, c-Ha-*ras*, mammary cells

The epidermal growth factor (EGF) superfamily of proteins includes several peptide mitogens such as EGF, transforming growth factor α (TGFα), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), cripto, epiregulin, and betacellulin (BC) [Plowman et al., 1990a; Higashiyama et al., 1992; Sasada et al., 1993; Brandt et al., 1994; Normanno et al., 1994a; Toyoda et al., 1995]. EGF, TGFα, AR, HB-EGF, epiregulin, and BC are peptides that bind exclusively to the EGF receptor (EGFR) [Sasada et al., 1993; Normanno et al., 1994a]. Rat Neu differentiation factor (NDF) and the human homolog, heregulins (HRGs) constitute the neuregulin subfamily of EGF-related peptides that were originally isolated from Ha-*ras* transformed EJ Rat-1 fibroblasts and from human MDA-MB-231 breast cancer cells, respectively [Yarden and Peles, 1991; Holmes et al., 1992; Peles et al., 1992; Wen et al., 1992, 1994; Peles and Yarden, 1993; Kung et al., 1994]. There are at least 15 different cell-associated and secreted isoforms of HRG that are derived by alternative splicing from a single gene and that fall into two major groups, α and β [Peles and Yarden, 1993; Wen et al., 1994]. Peptides that are also members of the neuregulin subfamily include the glial growth factors and acetylcholine receptor-inducing activity [Falls et al., 1993;

Abbreviations used: ADG, anchorage-dependent growth; AIG, anchorage-independent growth; AR, amphiregulin; BC, betacellulin; EGF, epidermal growth factor; EGFR, EGF receptor; HB-EGF, heparin-binding EGF-like growth factor; HRG, heregulin; NDF, Neu differentiation factor; TGFα, transforming growth factor α.

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Goodearl et al., 1993; Marchionni et al., 1993; Chen et al., 1994; Pinkas-Kramarski et al., 1994; Shah et al., 1994]. HRGs are heparin-binding glycoproteins that contain an immunoglobulin (Ig)-like domain and a common EGF-like repeat that differs between the α and β isoforms at the last disulfide loop of the EGF domain [Holmes et al., 1992; Wen et al., 1992, 1994; Peles and Yarden, 1993; Lu et al., 1995a,b]. In addition, the α and β isoforms differ at the juxtamembrane region and at the C-terminal cytoplasmic tail in the precursor. Most isoforms of HRG lack a hydrophobic signal peptide, contain NH_2-terminal nuclear localization sequences, and are initially processed from a larger transmembrane precursor [Holmes et al., 1992; Wen et al., 1994]. HRGs are mitogenic for some human breast cancer cells and for Schwann cells [Holmes et al., 1992; Goodearl et al., 1993; DeCorte et al., 1994; Kung et al., 1994]. However, in other breast cancer cells HRGs are growth-inhibitory and can induce the expression of milk proteins while in glial cells, and in astrocytes HRGs can promote differentiation and survival [Peles et al., 1992; Bacus et al., 1993, 1994; Peles and Yarden, 1993; Pinkas-Kramarski et al., 1994; Shah et al., 1994]. HRGs bind to either c-*erb* B-3 or c-*erb* B-4, which can in turn indirectly activate the c-*erb* B-2 tyrosine kinase through heterodimer formation and subsequent transphosphorylation [Kraus et al., 1989; Plowman et al., 1993a,b; Carraway et al., 1994; Carraway and Cantley, 1994; Culouscou et al., 1994; Sliwkowski et al., 1994].

Although HRG expression has been detected in several different human carcinoma cell lines and in a number of rat and human embryonic and adult tissues [Holmes et al., 1992; Wen et al., 1992; Peles and Yarden, 1993; Bacus et al., 1994; DeCorte et al., 1994; Kung et al., 1994; Pinkas-Kramarski et al., 1994], there is no information on the biological effects of HRG on nontransformed or oncogene-transformed mammary epithelial cells or on changes in HRG expression following transformation of epithelial cells with different oncogenes. In this report, we demonstrate that recombinant HRGβ1 differentially stimulates the anchorage-dependent growth (ADG) and anchorage-independent growth (AIG) of nontransformed human MCF-10A mammary epithelial cells compared to Ha-*ras* or c-*erb* B-2 transformed MCF-10A cells. In addition, it is shown that c-Ha-*ras* or c-*erb* B-2 transformed mouse NOG-8 mammary epithelial cells and transformed human MCF-10A cells express elevated levels of HRG mRNA and immunoreactive HRG.

MATERIALS AND METHODS

Cell Culture and Growth

Human mammary epithelial MCF-10A cells and c-Ha-*ras* (clone T2B) or c-*erb* B-2 (clone 8) transformed MCF-10A cells were grown as previously described [Ciardiello et al., 1990; Normanno et al., 1994b]. Mouse mammary NOG-8 epithelial cells and NOG-8 cells transformed with rat c-*neu* (clone 4 [Cl 4] and 8 [Cl 8]), with a point-mutated c-*neu* gene (*neu*T) or with a v-Ha-*ras* oncogene (SR2 *ras*), were grown as previously described [Ciardiello et al., 1989]. For ADG experiments, 3×10^4 nontransformed or transformed MCF-10A cells were seeded in six-well cluster dishes (Beckton Dickson Inc., Lincoln Park, NJ). After 48 h, cells were washed twice with phosphate-buffered saline (PBS) and switched to PC-1 serum-free medium (Hycor Biomedical Inc., Irvine, CA) that contained transferrin (10 μg/ml) and insulin (15 μg/ml) in the absence or in the presence of different concentrations of recombinant $rHRG\beta1_{177-244}$ (Genentech Inc., San Francisco, CA). Recombinant $rHRG\beta1_{177-244}$ contains the EGF-like domain and immediate flanking region, which is responsible for receptor binding and for the biological activity [Holmes et al., 1992; Carraway et al., 1994; Sliwkowski et al., 1994]. After 1–6 days of treatment with the rHRGβ1, cells were trypsinized and counted using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). For AIG assays, 3×10^4 cells were seeded in 0.5 ml of 0.3% agar supplemented with medium containing 10% fetal calf serum in the absence or in the presence of different concentrations of rHRGβ1. This suspension was layered over 0.5 ml of 0.8% agar medium base layer in 24-well cluster dishes (Costar, Cambridge, MA). After 14 days, colonies were stained with nitro blue tetrazolium, and colonies larger than 50 μm were counted with an Artek 880 colony counter (Artek Systems, Farmingdale, NY) as previously described [Normanno et al., 1994b].

RNA Isolation and Northern Blot Analysis

Total cellular RNA was extracted by lysis of cells in guanidine isothiocyanate and centrifuged over a cesium chloride cushion as previously described [Ciardiello et al., 1990; Nor-

manno et al., 1994b]. Ten to twenty micrograms of poly $(A)^+$ RNA was electrophoresed through a denaturing 1.2% agarose–2.2 M formaldehyde gel. Ethidium bromide staining of the gels showed that each lane contained an equivalent amount of RNA. The gels were then transferred to Biotrans nylon membranes (ICN Biomedicals, Costa Mesa, CA) and hybridized to the following ^{32}P-labeled nick-translated cDNA probes: 1.9 kb cDNA inserts containing the coding region for either rat NDF or human HRG (kindly provided by Duanazhi Wen, Amgen Inc., Thousand Oaks, CA) [Wen et al., 1992; Normanno et al., 1993]; a 0.5 kb *Hind*III / *Bam*HI human HRG cDNA fragment [Holmes et al., 1992]; a 1.7 kb *Eco*R1/*Hind*III cDNA fragment containing most of the extracellular domain, the transmembrane domain, and a part of the intracellular tyrosine kinase domain of human *erb* B-3 [Plowman et al., 1990b]; and a 4.4 kb full-length cDNA clone or a 630 bp (nucleotide positions 1707 to 2337) fragment of human *erb* B-4 [Plowman et al., 1993a] or a 770 bp human β-actin cDNA probe (Oncor, Gaithersburg, MD).

Western Blot Analysis

Protein lysates (50 μg per sample) were separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis using either 6% gels or 8–16% gradient gels (Novex, San Diego, CA), transferred to nitrocellulose, blocked with 3% dry milk, and reacted with a 1:2,000 to 1:1,000 dilution of a sheep IgG antibody raised against a recombinant *E. coli*–derived, full-length human HRGβ1 protein [Holmes et al., 1992] or with a comparable dilution of nonimmune sheep IgG or immune serum that had been preabsorbed with 10 μg/ml of recombinant $rHRG\beta1_{177-244}$. Immunoreactive proteins were visualized by enhanced chemiluminescence detection (Amersham, Arlington Heights, IL) with a 1:5,000 dilution of goat antisheep IgG conjugated to horseradish peroxidase.

RESULTS

HRGβ1 Stimulates the Growth of MCF-10A Cells

MCF-10A human mammary epithelial cells require exogenous EGF in conjunction with insulin or insulin-like growth factor-1 (IGF-1) for ADG [Ciardiello et al., 1990; Normanno et al., 1994b; Ram et al., 1995]. In the absence of EGF, MCF-10A cells are unable to proliferate in serum-free PC-1 medium that contains insulin (15 μg/ml) and enter a G1/G0 arrest [Normanno et al., 1994b]. The ADG requirement for EGF can be supplanted by other peptides that activate the EGFR such as TGFα or AR, which are equipotent as EGF [Normanno et al., 1994b]. In the absence of exogenous EGF, $rHRG\beta1_{177-244}$ (1 nM) was found to moderately stimulate the ADG or MCF-10A cells by approximately 35–50% over a 6 day growth period in serum-free, insulin-supplemented PC-1 medium (Fig. 1A). Under these same conditions, $rHRG\alpha_{177-239}$ (supplied by Berlex Biosciences, Richmond, CA) produced only a 10–15% growth stimulation (data not shown). In contrast to the nontransformed, parental MCF-10A cells, rHRGβ1 (1 nM) was ineffective in stimulating the ADG in serum-free, insulin-supplemented PC-1 medium of both the MCF-10A T2B *ras* and MCF-10A Cl 8 *erb* B-2 transformed cells (Fig. 1B,C). To ascertain the optimum growth-promoting concentration of peptide, cells were maintained for 4 days in serum-free, insulin-supplemented PC-1 medium with different amounts (10 pM–10 nM) of rHRGβ1 (Fig. 1D–F). The parental MCF-10A cells demonstrated a dose-dependent increase in growth in response to rHRGβ1 with an EC_{50} of ~100 pM and with a maximum 50% growth stimulatory effect (Fig. 1D). However, the Ha-*ras* and *erb* B-2 MCF-10A transformants were generally refractory under ADG conditions to all concentrations of rHRGβ1 (Fig. 1E,F).

MCF-10A parental cells fail to grow in soft agar in response to either exogenous EGF, TGFα, or AR, while Ha-*ras* and *erb* B-2 transformed MCF-10A cells grow in agar in both serum-free, insulin-supplemented PC-1 medium and in serum-containing medium in the presence of either EGF or TGFα but not AR [Normanno et al., in press]. In fact, the AIG of both transformed cell lines depends upon the presence of exogenous EGF or TGFα. rHRGβ1 produced a significant dose-dependent three- to fourfold stimulation in the agar growth of the nontransformed MCF-10A cells and the MCF-10A *erb* B-2 Cl 8 cells (Fig. 1G,I) with an EC_{50} ~20–40 pM. The AIG of the Ha-*ras* transformed MCF-10A cells was also stimulated by rHRGβ1 but to a lesser extent, producing only a 50% increase at ~1–2 nM (Fig. 1H).

Expression of HRG mRNA and Protein in Transformed MCF-10A and NOG-8 Cells

To ascertain if nontransformed, immortalized mouse or human mammary epithelial cells ex-

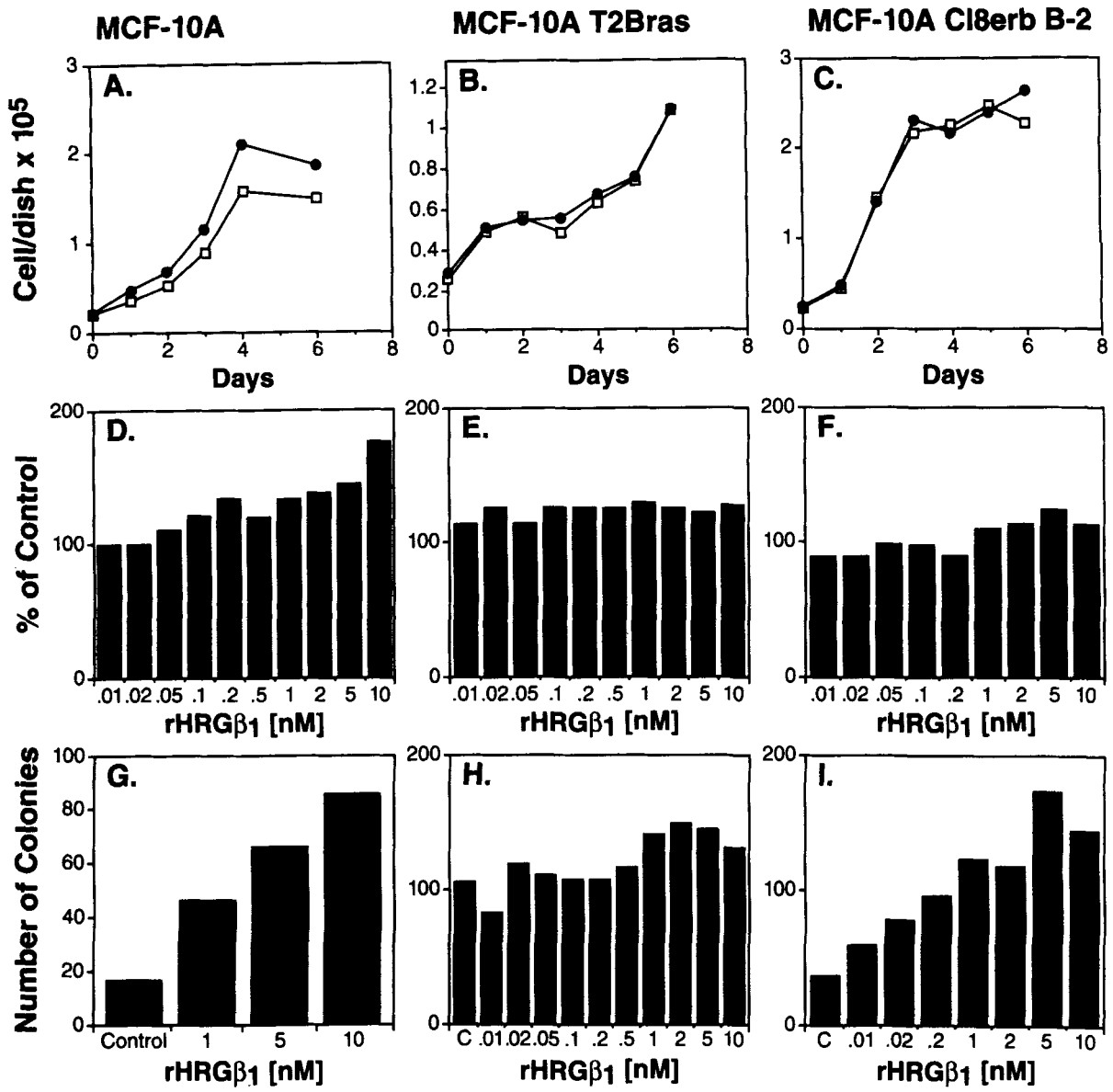

Fig. 1. Effect of recombinant rHRGβ1 on the ADG and AIG of MCF-10A, MCF-10A T2 *ras*, and MCF-10A Cl 8 *erb* B-2 human mammary epithelial cells. **A–C:** For ADG, cells were grown in serum-free medium with (●—●) or without (□—□) 1 nM rHRGβ1 for 1–6 days and counted daily. **D–F:** For ADG, cells were grown for 4 days in serum-free medium with different concentrations of rHRGβ1. **G–I:** For AIG, cells were grown in soft agar for 14 days with different concentrations of rHRGβ1, after which colonies were stained and counted. Results are the average of at least two separate experiments with triplicate determinations for each sample that did not vary by more than 10%.

press endogenous NDF/HRG mRNA, poly (A)$^+$ RNA was obtained from NOG-8 mouse mammary epithelial cells and from MCF-10A human mammary epithelial cells and screened for HRG mRNA expression by Northern blot hybridization using either a labeled rat NDF cDNA probe for the mouse NOG-8 cells or a human HRG cDNA probe for the human MCF-10A cells (Fig. 2A,B). Neither parental NOG-8 (Fig. 2A) nor parental MCF-10A (Fig. 2B) mammary epithelial cells express significant levels of NDF/HRG mRNA that can be detected by Northern blot analysis. In contrast, two c-*neu* transformed clones of NOG-8 (Cl 4 and Cl 8), a point-mutated *neu* NOG-8 transformed clone (*neu T*), and a v-Ha-*ras* transformed NOG-8 clone (SR2 *ras*) exhibited very high levels of NDF/HRG mRNA transcripts of 2.5 kb, 6.5 kb, and 8.5 kb as compared to HRG mRNA levels that were detected in human MDA-MB-231 breast cancer

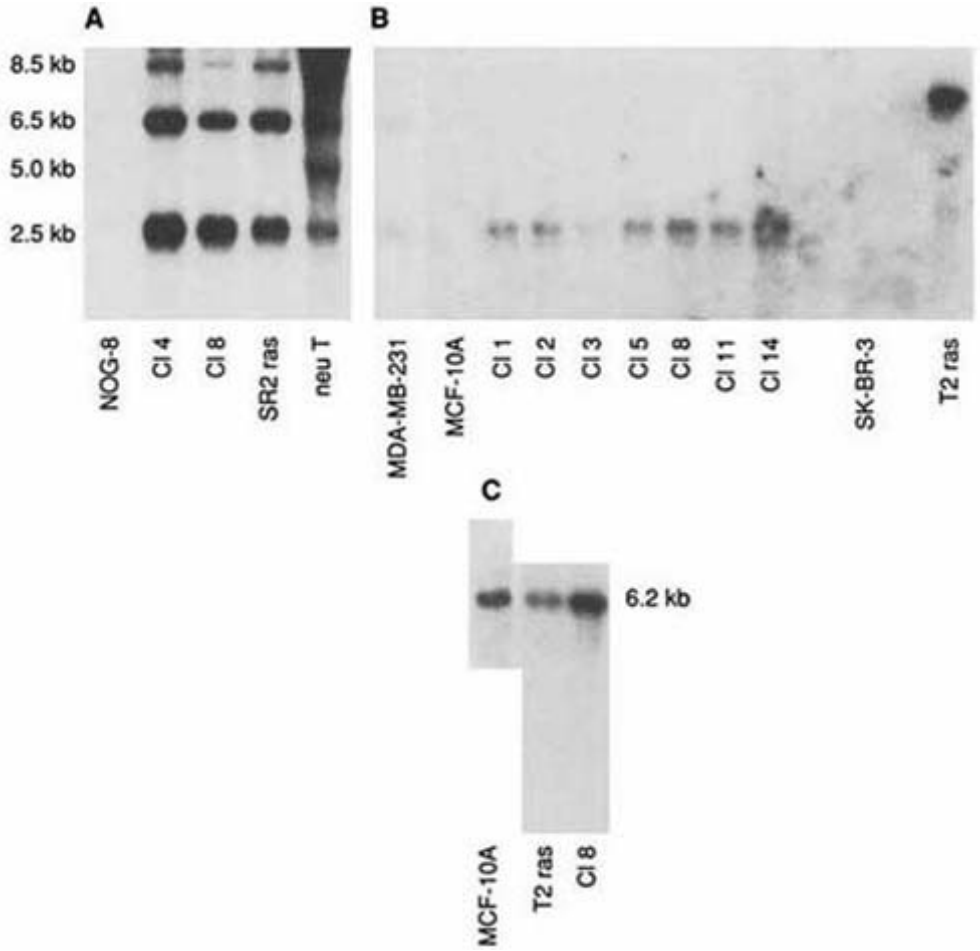

Fig. 2. Northern blot of NDF/HRG and *erb* B-3 mRNA expression in parental and transformed mouse NOG-8 and human MCF-10A mammary epithelial cells. Twenty micrograms of poly (A)$^+$ RNA obtained from **(A)** mouse NOG-8, NOG-8 c-*neu* transformed clones (Cl 4 and Cl 8) cells, NOG-8 *neu* T transformed cells, or SR2 *ras* transformed NOG-8 cells or **(B)** from human MDA-MB-231 breast cancer cells, human MCF-10A mammary epithelial cells, c-erb B-2 transformed MCF-10A clones (Cl 1, 2, 3, 5, 8, 11, 14), Ha-ras (T2 *ras*) transformed MCF-10A cells, or SK-BR-3 human breast cancer cells were electrophoresed, Northern blotted, and probed with either a ^{32}P-labeled rat NDF cDNA (A) or a human HRG cDNA (B) probe. **C:** Poly (A)$^+$ RNA obtained from MCF-10A, T2 *ras* transformed, or erb B-2 Cl 8 transformed MCF-10A cells were processed as above and probed with a ^{32}P-labeled human c-*erb* B-3 probe.

cells that express only the 2.5 kb and 6.5 kb HRG mRNA transcripts (Fig. 2A). In addition, NOG-8 *neu* T cells express a fourth transcript of ~5 kb. Analysis of Northern blots of poly(A)$^+$ RNA that was obtained from oncogene transformed MCF-10A cells demonstrated that several c-*erb* B-2 transformed clones of MCF-10A cells exhibited moderate levels of expression of a corresponding 2.5 kb HRG mRNA transcript, while T2B *ras* MCF-10A cells possess a unique 6.8 kg HRG mRNA transcript (Fig. 2B).

To ascertain if the presence of NDF/HRG mRNA that is observed in the transformed MCF-10A clones is also reflected by a corresponding detection of immunoreactive isoforms of HRG in these cells, cell lysates from these cells were subjected to Western blot analysis after separation on either an 8–16% gradient SDS denaturing gel to more fully resolve low molecular weight, immunoreactive proteins (Fig. 3A) or a 6% gel to separate moderate to high molecular weight, immunoreactive proteins (Fig. 3C). Detection of immunoreactive isoforms of HRG was accomplished by using an anti-HRG sheep antibody that was generated against a full-length recombinant human HRGβ1 protein. Specific proteins of 25, 29, 50, and 115 kDa could be detected in varying amounts in lysates prepared from human MDA-MB-231 breast cancer cells and from the transformed MCF-10A T2B *ras* and MCF-10A *erb* B-2 Cl 8 cells but not in lysates prepared from HRG mRNA negative SK

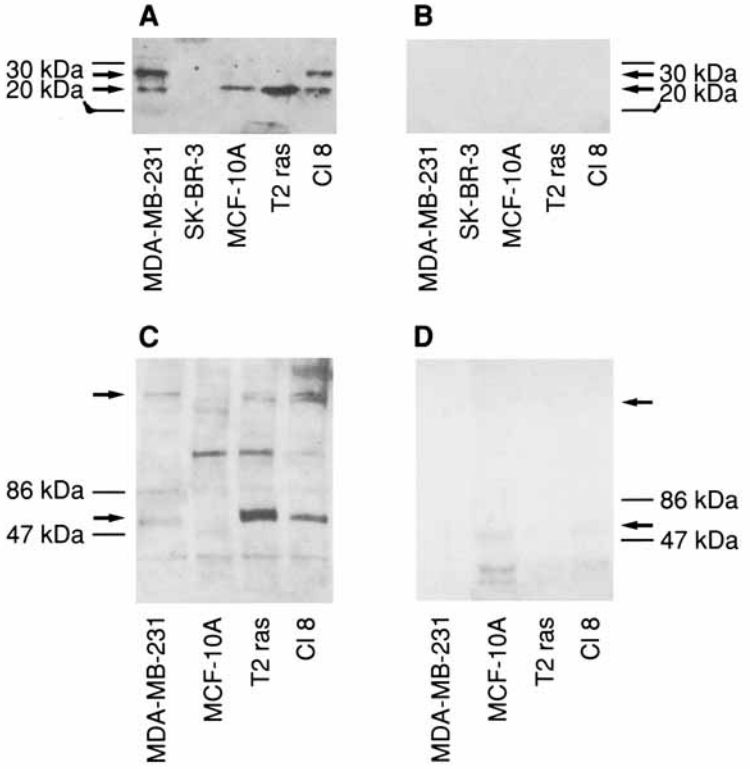

Fig. 3. Western blot of immunoreactive HRG proteins in parental and transformed MCF-10A human mammary epithelial cells. Cell lysates were prepared from human MCF-10A mammary epithelial cells, from *erb* B-2 Cl 8, from the T2 *ras* transformed MCF-10A cells, or from breast cancer MDA-MB-231 or SK-BR-3 cells, electrophoresed through an 8–16% SDS gel (**A,B**) or through a 6% SDS gel (**C,D**), blotted, and reacted with a sheep anti-HRG antibody (A,C) or with nonimmune sheep serum (B,D). *Arrows* indicate positions of specific immunoreactive proteins.

BR-3 breast cancer cells [Holmes et al., 1992; Wen et al., 1994] (Fig. 3A,C). Although the nontransformed parental MCF-10A cells expressed a 25 kDa immunoreactive species, other HRG isoforms were not detected in these cells (Fig. 3A). There were several nonspecific protein bands in all of the cell lysates that were detected by both the sheep anti-HRG antiserum and by the nonimmune sheep serum (Fig. 3B,D). This was especially true for proteins above 45 kDa (Fig. 3D). However, detection of the 25, 29, 50, and 115 kDa proteins by the antibody was specific since the nonimmune sheep IgG was unable to detect similar proteins in the same lysates and since preabsorption of the antibody with rHRGβ1 abrogated detection of these proteins (Fig. 3B,D).

MCF-10A cells express ~2–3 × 10^5 EGFR sites/cell and ~ 10^4 *erb* B-2 sites/cell [Ciardiello et al., 1990]. Following transformation with either c-Ha-*ras* or with c-*erb* B-2, there is little change in EGFR levels, while in the *erb* B-2 overexpressing clones there is a ten- to twenty-fold increase in the number of *erb* B-2 sites/cell [Ciardiello et al., 1990]. Since c-*erb* B-3 or c-*erb* B4 function as high affinity receptors for different isoforms of NDF/HRGs via heterodimerization with c-*erb* B-2 [Plowman et al., 1993b; Culouscou et al., 1994; Carraway et al., 1994; Sliwkowski et al., 1994; Carraway and Cantley, 1994], MCF-10A cells and several of the transformed clones were examined for *erb* B-3 and *erb* B-4 mRNA expression. A 6.2 kb *erb* B-3 mRNA transcript could be detected in the MCF-10A cells, in the *erb* B-2 Cl 8 cells, and in the T2 *ras* clone by Northern blot hybridization (Fig. 2C). In contrast, no *erb* B-4 mRNA could be detected in any of the MCF-10A cell lines.

DISCUSSION

The present study demonstrates for the first time that transformation of immortalized mouse and human mammary epithelial cells by a point-mutated c-Ha-*ras* gene or by overexpression of a c-*erb* B-2 or a point-mutated c-*neu* or overexpression of a normal c-*neu* proto-oncogene induces the expression of multiple NDF/HRG mRNA transcripts and different immunologically detect-

able isoforms of HRG protein. The results are in accord with the observation that enhanced expression of NDF occurs after transformation of Rat-1 fibroblasts with a point-mutated c-Ha-*ras* gene or after transformation of hamster fibroblasts with an activated *cph* gene [Yarden and Peles, 1991; Avila et al., 1995]. In *ras* transformed Rat-1 fibroblasts, in mouse Balb/MK keratinocytes, and in different rat and human tissues, HRG mRNA transcripts of 2, 2.5, 3, 3.5, 6.5, 7, 9, 10, and 12 kb have been detected in various ratios and intensities [Holmes et al., 1992; Wen et al., 1992; Falls et al., 1993; Marchionni et al., 1993; Chen et al., 1994; Corfas et al., 1995; Marikovsky et al., 1995]. It has been suggested that the 2 kb transcript encodes soluble isoforms of HRG, while the larger transcripts potentially encode other membrane-associated HRG isoforms [Chen et al., 1994]. Differences in alternative mRNA splicing could account for the presence of at least three distinct transcripts in the transformed NOG-8 cells and a fourth transcript in the NOG-8 *neu* T cells [Falls et al., 1993]. In this regard, there are at least 14 different mammalian NDF/HRG mRNAs representing nine distinct splicing patterns, which could account for the variations in mRNA sized transcripts in these transformed mammary epithelial cells [Marchionni et al., 1993].

Since normal breast epithelium and nearly 90% of human breast carcinoma cells express *erb* B-3 [Lemoine et al., 1992], since approximately 25–30% of primary human breast tumors express HRG mRNA, and since amplification and/or overexpression of c-*erb* B-2 has been detected at an equal frequency in invasive breast carcinomas that are rapidly growing and extremely aggressive [Bacus et al., 1993, 1994; Dougall et al., 1994; Normanno et al., 1995], these findings have potential clinical significance with respect to ligands that may be amplifying the tyrosine kinase and transforming activity of *erb* B-2 through binding to *erb* B-3. The data also demonstrate that overexpression of NDF/HRGs in transformed mammary epithelial cells may have functional significance as a growth factor since picomolar concentrations of rHRGβ1 but not rHRGα are able to moderately stimulate the serum-free ADG of nontransformed MCF-10A human mammary epithelial cells in the presence of insulin, although to a lesser degree than either EGF, TGFα, or AR [Ciardiello et al., 1990; Normanno et al., 1994b]. Recently, Ram et al. [1995] have also demonstrated that human recombinant isoforms of NDF/HRG can induce the transphosphorylation of c-*erb* B-2 in MCF-10A cells and that various isoforms of HRGβ but not of HRGα could substitute for either EGF, insulin, or IGF-1 in stimulating the serum-free, ADG of these cells. Likewise, they also demonstrated that either the α or β isoforms of HRG could stimulate the ADG of the c-*erb* B-2 overexpressing MCF-10A Cl 8 cells in serum-free and insulin/IGF-free medium. The present study extends these observations since, like exogenous EGF or TGFα, rHRGβ1 was able to significantly stimulate the AIG of the *erb* B-2 transformed MCF-10A cells and to a lesser extent the MCF-10A *ras* transformants [Normanno et al., 1994b]. More importantly, this study demonstrates that rHRGβ1 is unique amongst other EGF-like peptides since it can induce the AIG of nontransformed MCF-10A cells, which is one characteristic of a transformed phenotype. This may be related to the unique ability of HRGβ1 but not other members of the EGF family of peptide mitogens to simultaneously function as both an EGF-like and IGF-like ligand [Ram et al., 1995]. Finally, Marikovsky et al. [1995] have recently shown that in EGF-dependent mouse Balb/MK keratinocytes different isoforms of NDFβ but not NDFα can replace EGF as a mitogen and that this response correlates with the binding affinities of these different isoforms to the *erb* B-3 protein that is expressed in these cells. Similar to the data presented in this study with MCF-10A mammary epithelial cells, Marikovsky et al. [1995] also found that different NDFβ isoforms were generally two- to threefold less potent than EGF in stimulating Balb/MK keratinocyte ADG under serum-restricted conditions, producing only a 1.5- to twofold increase in proliferation.

Multiple isoforms of HRG have been described in the conditioned medium and from cell lysates of HRG mRNA positive human carcinoma cells, from *ras* and *cph* transformed fibroblasts, and from COS-7 cells that are transiently expressing rat pro-NDF [Yarden and Peles, 1991; Holmes et al., 1992; Goodearl et al., 1993; DeCorte et al., 1994; Kung et al., 1994; Wen et al., 1994; Avila et al., 1995]. These mosaic glycoproteins range in molecular weight from 25–115 kDa. The size heterogeneity is due to differences in N- and O-linked glycosylation and to variations in proteolytic N- and C-terminal processing of the membrane-associated precursor [Lu

et al., 1995a,b]. The MCF-10A transformants synthesize major 50 and 115 kDa isoforms of HRG and lesser amounts of 25 and 29 kDa isoforms. The smaller species may represent underglycosylated species of HRG since bacterially derived human HRGα has an apparent molecular weight of ~30 kDa [Lu et al., 1995a]. Similarly, the larger 115 and 50 kDa HRG species are analogous in size to Chinese hamster ovary (CHO) cell-derived forms of NDF [Lu et al., 1995b]. Similar specific immunoreactive proteins were detected in HRG mRNA positive MDA-MB-231 breast cancer cells but not in HRG mRNA negative SK-BR-3 cells [Holmes et al., 1992; Wen et al., 1994]. These results collectively suggest that one of the several different isoforms of HRG that are expressed by the *ras* and *erb* B-2 transformed MCF-10A mammary epithelial cells could function in an autocrine and/or intracrine fashion to regulate the growth of these cells either alone or in combination with other endogenously produced EGF-related peptides. Such a possibility has been formally demonstrated in *cph* transformed hamster fibroblasts using an NDF neutralizing antibody [Avila et al., 1995]. In this context, *ras* transformation of either NOG-8 or MCF-10A mammary epithelial cells leads to an upregulation in the expression of endogenous TGFα and AR, while *erb* B-2 or *neu* transformation leads only to an increase in AR expression [Ciardiello et al., 1989, 1990; Normanno et al., 1994b]. In fact, endogenous TGFα and AR can cooperate via cross-induction in their ability to enhance the growth of these MCF-10A and NOG-8 transformants through external autocrine- and internal intracrine-dependent pathways involving the EGFR, respectively [Ciardiello et al., 1989, 1990, 1992; Barnard et al., 1994; Normanno et al., 1994b]. Conversely, NDF/HRG may indirectly inhibit ligand binding to the EGF receptor by a negative transregulatory mechanism in breast cancer cells that are expressing low levels of the EGF receptor and *erb* B-2 [Karunagaran et al., 1995].

MCF-10A cells express *erb* B-2 and EGFR [Ciardiello et al., 1990]. The present study and those of Ram et al. [1995] demonstrate that MCF-10A cells and their transformants also express *erb* B-3, which is expressed in a majority of infiltrating ductal breast carcinomas [Kraus et al., 1989; Lemoine et al., 1992; Bacus et al., 1994]. However, these cells fail to express *erb* B-4 as assessed by either Northern blot analysis or by more sensitive RT-PCR [Ram et al., 1995]. This is probably functionally significant since *erb* B-3 can serve as a high affinity receptor for HRG that exhibits an enhanced tyrosine kinase activity after HRG-induced heterodimerization with *erb* B-2 [Carraway et al., 1994; Kita et al., 1994; Sliwkowski et al., 1994]. In fact, coexpression of $p180^{erb\ B\text{-}3}$ and $p180^{erb\ B\text{-}2}$ and formation of an *erb* B-2–*erb* B-3 complex after ligand binding is obligatory for HRG-induced growth and for HRG-induced phosphorylation of both proteins [Carraway et al., 1995; Marikovsky et al., 1995]. In MCF-10A cells, HRGβ1 but not HRGα was able to significantly stimulate ADG and AIG. A similar observation has been made with different CHO cell-derived isoforms of HRGβ and HRGα. HRGβ but not HRGα isoforms were able to significantly stimulate the growth of NIH3T3 cells that were overexpressing c-*erb* B-3 [Lu et al., 1995b]. This result is probably due to the lower binding affinities of the HRGα isoforms compared to the HRGβ isoforms for interaction with *erb* B-3 [Lu et al., 1995b]. The EGFR can also heterodimerize with either *erb* B-2 or *erb* B-3 after ligand binding, and this heterodimerization can also lead to an enhancement in EGFR, *erb* B-2, and *erb* B-3 tyrosine kinase activities through reciprocal intermolecular transphosphorylations [Dougall et al., 1993, 1994; Bacus et al., 1994; Carraway and Cantley, 1994; Kita et al., 1994; Sliwkowski et al., 1994; Soltoff et al., 1994; Carraway et al., 1995]. Therefore, various ligand-induced heterodimeric combinations between the EGFR, *erb* B-2, *erb* B-3, and/or *erb* B-4 may provide a mechanism for signal amplification at low concentrations of particular sets of EGF-like ligands [Dougall et al., 1993, 1994; Bacus et al., 1994; Carraway and Cantley, 1994; Culouscou et al., 1994; Sliwkowski et al., 1994] and probably accounts for the synergy that is observed between overexpression of the EGFR *erb* B-2 and *erb* B-3 in the transformation of rodent fibroblasts [Dougall et al., 1993, 1994; Alimandi et al., 1995]. This same phenomenon may also contribute to the autocrine-dependent growth of oncogene transformed mammary epithelial cells through the production of multiple EGF-related growth factors, which are able to function through different yet interacting receptors of the *erb* B family [Ciardiello et al., 1990, 1992; Bacus et al., 1994; Normanno et al., 1994a,b; Qi et al., 1994; Alimandi et al., 1995].

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