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Notch4 and Wnt-1 Proteins Function to Regulate Branching Morphogenesis of

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an Opposing Fashion

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Elongation and branching of epithelial ducts is a crucial event during the development of the mammary gland. Branching morphogenesis of the mouse mammary epithelial TAC-2 cell line was used as an assay to examine the role of Wnt, HGF, TGF-β, and the Notch receptors in branching morphogenesis. Wnt-1 was found to induce the elongation and branching of epithelial tubules, like HGF and TGF-β2, and to strongly cooperate with either HGF or TGF-β2 in this activity. Wnt-1 displayed morphogenetic activity in TAC-2 cells as it induced branching even under conditions that normally promote cyst formation. The Notch4(int-3) mammary oncoprotein, an activated form of the Notch4 receptor, inhibited the branching morphogenesis normally induced by HGF and TGF-β2. The minimal domain within the Notch4(int-3) protein required to inhibit morphogenesis consists of the CBF-1 interaction domain and the cdc10 repeat domain. Coexpression of Wnt-1 and Notch4(int-3) demonstrates that Wnt-1 can overcome the Notch-mediated inhibition of branching morphogenesis. These data suggest that Wnt and Notch signaling may play opposite roles in mammary gland development, a finding consistent with the convergence of the wingless and Notch signaling pathways found in *Drosophila.* © 1998 Academic Press

Key Words: **Notch4; int-3; Wnt-1; mammary oncogene; branching morphogenesis.**

INTRODUCTION

The development of the murine mammary gland involves an intricate sequence of proliferative, morphogenetic, and differentiative events, which gradually results in the formation of an arborized tree-like structure of epithelial ducts. Postnatal development of the mammary gland is influenced by gonadal hormones, with distinct developmental stages occurring during puberty, estrous, pregnancy, and lactation. At birth, the mammary epithelial ducts have few side-branches. During puberty, the epithelial ducts rapidly elongate and branch, and give rise to a highly organized epithelial structure with terminal end buds and lateral buds. The terminal end buds are the major sites of proliferation, whereas the lateral buds differentiate into alveoli during each estrous cycle. During pregnancy, the alveoli rapidly increase in size and number resulting in the development of fully differentiated lobules, which will produce milk at lactation. The mammary gland remodels after lactation ceases, and this process is characterized by the involution of the secretory lobules and regression to the ductal tree observed at puberty (reviewed in Daniel and Silberstein, 1987; Pitelka *et al.,* 1973; Russo *et al.,* 1989).

Mesenchymal–epithelial and epithelial–epithelial interactions are essential in the regulation of growth and development of the murine mammary gland. Peptide growth factors, such as epidermal growth factor (EGF) (Haslam *et al.,* 1992), fibroblast growth factors (FGF) (Coleman-Krnacik and Rosen, 1994), hepatocyte growth factor (HGF), insulin-like growth factor II (IGF-II) (Bates *et al.,* 1995), neuregulin (NRG) (Yang *et al.,* 1995), and transforming growth factor-β (TGF-β) (Daniel *et al.,* 1996; Pierce *et al.,* 1993), have been implicated as regulators of mammary gland development

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based on their expression patterns and, in some cases, on their abilities to affect the development of the mammary gland. HGF (or scatter factor) is expressed in the mammary mesenchyme during ductal branching, whereas its tyrosine kinase receptor c-met is expressed in the mammary epithelial ducts at all stages (Niranjan *et al.,* 1995). HGF can promote branching morphogenesis of the mammary ductal tree (Niranjan *et al.,* 1995; Pepper *et al.,* 1995; Soriano *et al.,* 1995; Yang *et al.,* 1995) in several experimental settings. TGF-β1 is expressed in the epithelial compartment of the mammary gland at all stages, except during lactation (Daniel *et al.,* 1996; Smith, 1996). *In vivo,* TGF-β1 has been shown to inhibit ductal outgrowth from the mammary end buds (Kordon *et al.,* 1995; Pierce *et al.,* 1993). *In vitro,* however, TGF-β1 has been shown to induce opposite effects depending on its concentration. TGF-β1 at high concentrations (0.5–5 ng/ml) inhibits ductal elongation and branching of TAC-2 mammary epithelial cells, whereas at low concentrations (5–100 pg/ml) it is able to stimulate these biological processes (Soriano *et al.,* 1996).

The Wnt family of secreted growth factors are also implicated as regulators of the developing mouse mammary gland (Nusse and Varmus, 1992). *Wnt* genes are expressed during ductal development of the gland (*Wnt*-2, *Wnt*-5a, *Wnt*-7, and *Wnt*-10b) and during lobular development at pregnancy (*Wnt*-4, *Wnt*-5b, and *Wnt*-6), and the expression of most *Wnt* transcripts is downregulated during lactation (Gavin and McMahon, 1992; Weber-Hall *et al.,* 1994). This pattern of expression during periods of morphogenesis has led to a proposed role for *Wnt* genes in morphogenetic events during mammary gland development. *Wnt* gene expression has been documented in both the stromal and epithelial compartments of the mammary gland, raising the possibility of involvement in both stromal–epithelial and epithelial–epithelial interactions (Buhler *et al.,* 1993; Weber-Hall *et al.,* 1994). The *Wnt*-1 gene is not normally expressed within the mouse mammary gland; however, its expression can contribute to tumorigenesis when activated by insertion of mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary tumors (Nusse *et al.,* 1984). Mammary gland tumors develop in transgenic mice where ectopic *Wnt*-1 gene expression is controlled by the MMTV promoter; these mice display hyperplasia of the mammary epithelium and an increased incidence of tumors (Tsukamoto *et al.,* 1988).

The *Notch4* gene was also identified as a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors (Robbins *et al.,* 1992; Sarkar *et al.,* 1994). The *Notch4* gene encodes for a large transmembrane receptor protein (Gallahan and Callahan, 1997; Uyttendaele *et al.,* 1996). The int-3 oncoprotein is activated by MMTV insertion and corresponds to a truncated form of Notch4 which has most of its extracellular domain deleted (Uyttendaele *et al.,* 1996); this mutated version of Notch4 will be referred to as Notch4(int-3). In contrast to *Wnt*-1, expression of the *Notch4*(*int-3*) oncogene as a transgene in the mouse mammary gland results in impaired development of the mammary gland which no longer generates a tree-like structure of epithelial ducts. Instead, a hyperproliferative mass of undifferentiated epithelial cells is observed near the nipple, from which undifferentiated mammary carcinomas rapidly develop (Jhappan *et al.,* 1992).

The aim of this study was to define the roles of both Wnt and Notch signaling in mammary gland ductal morphogenesis. Using a previously described model in which TAC-2 mammary epithelial cells grown in collagen gels form branching cords or tubules in response to HGF or TGF-β1 (Soriano *et al.,* 1995, 1996), we demonstrate that activation of the Wnt and Notch signaling pathways has opposite effects on branching morphogenesis. Wnt-1 acts to induce branching morphogenesis, whereas Notch4(int-3) inhibits branching morphogenesis by either HGF or TGF-β. Wnt-1 has the capacity to overcome the Notch4(int-3)-mediated inhibition of branching morphogenesis.

MATERIALS AND METHODS

Reagents. Recombinant human HGF (rhHGF) was provided by Genentech, Inc. (San Francisco, CA). Recombinant TGF-β2 was provided by Dr. G. Gunderson (Columbia University, New York, NY). Rat tail collagen solution was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA monoclonal antibody (12CA5) was from Berkeley Antibody Co. (Richmond, CA) and HRP-conjugated sheep anti-mouse immunoglobulin G was from Amersham (Arlington Heights, IL).

cDNA clones. The murine *Notch4*(*int-3*) cDNA corresponds to a truncated *Notch4* cDNA, residues 4551 to 6244 of *Notch4* (Uyttendaele *et al.,* 1996). An oligonucleotide encoding the hemagglutinin (HA) antigenic determinant was appended to the 3′ end of the *Notch4*(*int-3*) and *Wnt*-1 cDNA's. Eighteen codons were added that specify the amino acid sequence SMAYPYDVPDYASLGPGP, including the nine residue HA epitope (underlined). HA-tagged *Notch4*(*int-3*) and *Wnt*-1 cDNAs were created by subcloning each cDNA into Bluescript KS (Stratagene) with the coding region of the HA epitope situated downstream of the newly inserted cDNA. These two sequences were made colinear by ''loop-out'' mutagenesis using oligonucleotides designed to eliminate the stop codon and noncoding 3′ sequence of the *Notch4*(*int-3*) and *Wnt*-1 cDNAs. Oligonucleotides used in this procedure are as follows: *Notch4*(*int-3*), CGG TTG TAA GAA ATC TGA ACT CCA TGG CCT ACC CAT ATG; *Wnt*-1, CGC GCG TTC TGC ACG AGT GTC TAT CCA TGG CCT ACC C. The 5′ end of each oligo is complementary to the C-terminus of *Notch4*(*int-3*) or *Wnt*-1 cDNA and their 3′ ends anneal to HA epitope-encoding sequence (underlined). Mutagenesis was carried out with the Muta-Gene *in vitro* mutagenesis kit (Bio-Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing. *Notch4*(*int-3*) cDNA deletion mutants were generated from the epitope-tagged *Notch4*(*int-3*) construct by restriction enzyme cloning, and were named ΔNT, ΔCDC, ΔCT, and ΔNTΔCT. The ΔNT deletion mutant corresponds to nucleotides 4921 to 6244 of the *Notch4* sequence. The ΔCDC deletion mutant corresponds to nucleotides 4551 to 4864 and to nucleotides 5706 to 6244 of the *Notch4* sequence. The ΔCT deletion mutant corresponds to nucleotides 4551 to 5718 of the *Notch4* sequence. The ΔNTΔCT deletion mutant corresponds to nucleotides 4921 to 5718 of the *Notch4* sequence.

Cell culture. The TAC-2 cell line was derived from NMuMG cells as described previously (Soriano *et al.,* 1995). TAC-2 cells were

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grown on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO). The BOSC 23 retrovirus packaging cell line (Pear *et al.,* 1993) was obtained from Dr. W. Pear (MIT, MA) and grown in DMEM containing 10% FCS. Both culture media were supplemented with penicillin (500 IU/ml) and streptomycin (100 μg/ml). Both cell lines were grown at 37°C in 8% CO_2.

Cell line generation. HA-tagged cDNAs were inserted into the retroviral vector pLNCX (Miller and Rosman, 1989) wherein neomycin phosphotransferase (neo) expression is controlled by the murine leukemia virus LTR, and cDNA transcription is controlled by the cytomegalovirus (CMV) enhancer/promoter. The retroviral vector pLHTCX was derived from pLNCX; however, the neo gene is replaced by a hygromycin-resistence gene. Populations of TAC-2 cells, expressing either HA-tagged *Notch4*(*int-3*) or *Wnt*-1 cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting constructs into the BOSC 23 cell line by calcium phosphate coprecipitation, as previously described (Pear *et al.,* 1993). Retroviral infection of TAC-2 cells was carried out by culturing cells with viral supernatants collected from transfected BOSC 23 cells 2 days posttransfection. Infections were carried out in the presence of 4 μg/ml polybrene for 12 h after which medium was replaced to DMEM + 10% FCS. One day postinfection the culture medium was replaced to DMEM + 10% FCS containing 500 μg/ml geneticin (GIBCO BRL Life Technologies, Grand Island, NY) or 200 μg/ml hygromycin B (Sigma Chemical Co.). Colonies appeared 5 days later and were pooled into medium containing 250 μg/ml geneticin or 200 μg/ml hygromycin B. These resultant populations, each composed of at least 50 clones, were used in assays described below.

Collagen cell culture assays. TAC-2 cell lines were harvested using trypsin–EDTA, centrifuged, and embedded in three-dimensional collagen gels as previously described (Soriano *et al.,* 1995). Briefly, 8 vol of rat tail collagen solution (approximately 1.5 mg/ml) were mixed with 1 vol of 10× minimal essential medium (GIBCO) and 1 vol of sodium bicarbonate (11.76 mg/ml) in a sterile tube kept on ice to prevent premature collagen gellation. TAC-2 cells were resuspended in the cold mixture at cell densities of 2 or 4×10^4 cells/ml and 0.5-ml aliquots were dispensed into 16-mm wells of 24 multiwell plates (Becton Dickinson Labware). After the collagen mixture had gelled, 1 ml of complete medium (DMEM + 10% FCS) with or without HGF or TGF-β2 was added to each well. TAC-2 collagen gel cultures were initially carried out in the presence and absence of 2 mM sodium butyrate, but since no difference in phenotypes was observed, the sodium butyrate was omitted in all experiments. Media were changed every 2 days, and after 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase-contrast microscope on Kodak T-Max film (100× magnification).

Quantification of cord length and branching. TAC-2 cells were suspended at 5×10^3 or 1×10^4 cells/ml in collagen gels (500 μl) cast into 16-mm wells of 4-well plates (Nunc, Kampstrup, Rosklide, Denmark) and incubated in 500 μl complete medium in the presence or the absence of 10 ng/ml HGF. After 7 days, the cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and at least three randomly selected fields (measuring 2.2 × 3.4 mm) per experimental condition in each of three separate experiments were photographed with a Nikon Diaphot TMD inverted photomicroscope. The total length of the cords present in each individual colony was measured with a Qmet 500 image analyzer (Leyca Cambridge Ltd., Cambridge, UK). Cord length was considered as "0" in: (a) colonies with a spheroidal shape, and (b) slightly elongated structures in which the length to diameter ratio was less than 2. Quantification of branching was performed by counting all branch points in each colony. Values of cord length and branching obtained from the largest colonies are an underestimate, since in these colonies a considerable proportion of cords were out of focus and therefore could not be measured. Values were expressed either as mean cord length and number of branch points per photographic field (Soriano *et al.,* 1996) or as mean cord length and number of branch points per individual colony (Soriano *et al.,* 1995). The mean values for each experimental condition were compared to controls using the Student unpaired *t* test.

Immunoblot analysis. HA-tagged Notch4(int-3), Notch4(int-3) deletion mutants and Wnt-1 proteins from lysates of TAC-2 cell populations were analyzed by immunoblotting. To maximize protein expression, TAC-2 cells were treated with 2 mM sodium butyrate for 16 h prior to lysis. Cells were washed twice with cold PBS and then removed from dishes in 1.5 ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 2000*g* at 4°C for 5 min and lysed in 90 μl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10,000*g* at 4°C for 10 min, and protein contents were determined using the Bio-Rad protein determination kit. Lysates containing 40 μg protein were electrophoresed in 10% SDS–polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose by electroblotting, and then blocked overnight at 4°C in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 1% bovine serum albumin (fraction V). Blots were then incubated in anti-HA monoclonal antibody (12CA5) diluted 1:100 in TBST at room temperature. After 4 h, the blot was washed three times for 5 min each in TBST. Blots were exposed to a 1:16,000 dilution of HRP-conjugated sheep anti-mouse IgG. Blots were washed as above and then incubated 1–2 min in enhanced chemiluminescence reagents (Amersham Inc., IL) and exposed to X-ray film (Fujifilm, Fuji Photo Film Co., LTD., Tokyo).

RESULTS

When suspended in collagen gels, TAC-2 mammary epithelial cells form small slowly growing colonies with a morphology ranging from irregular-shaped cell aggregates to poorly branched structures. Under these same conditions, TAC-2 cells grown in the presence of either HGF or TGF-β1 develop an extensive network of branching cords that consist of elongated epithelial cords or tubules with multiple branch points (Soriano *et al.,* 1995, 1996). This TAC-2 cell phenotype is thus reminiscent of the branching morphogenesis of epithelial ducts in the mammary gland and provides an experimental model to study the roles of growth factors and receptors in the development of the mammary gland. To investigate the role of Wnt and Notch signaling in mammary epithelial cell growth and morphogenesis, we ectopically expressed either the Wnt-1 or activated Notch4(int-3) oncoproteins and analyzed their effects on branching morphogenesis of TAC-2 cells. The results described below represent those found with several independently derived cell lines, including independent lines that were programmed to express proteins using a different promoter, as noted in the text.

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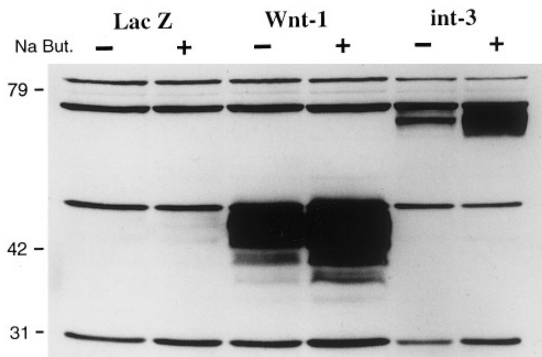

FIG. 1. Immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies. TAC-2 cells programmed to express LacZ, Wnt-1, or int-3 were grown in the presence or absence of sodium butyrate. Wnt-1 and int-3 proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment.

Wnt-1 Stimulates TAC-2 Cell Branching Morphogenesis

The biological activity of Wnt proteins was evaluated by generating TAC-2 cells ectopically expressing a *Wnt*-1 cDNA. TAC-2 cell lines programmed to express *Wnt*-1 (TAC-2 Wnt-1) were generated using the retroviral vector pLNCX to drive *Wnt*-1 expression from the CMV promoter. As a control, TAC-2 cells were generated that were programmed to express LacZ (TAC-2 LacZ). To evaluate the expression levels of Wnt-1 proteins in the cell lines generated, the *Wnt*-1 cDNA was fused at the carboxy terminus to the hemagglutinin-epitope (HA) tag, allowing us to detect Wnt-1 proteins in immunoblot analysis using the anti-HA monoclonal antibody (Fig. 1). Cell extracts from TAC-2 cell lines contained Wnt-1 proteins (Fig. 1) that migrated as a series of proteins with molecular weights between 41 and 45 kDa, due to differential glycosylation. The ectopic expression of Wnt-1 proteins in TAC-2 Wnt-1 cells can be significantly increased by treating cells with sodium butyrate (2 mM), which enhances transcription of the CMV promoter (Fig. 1). In order to evaluate the effects of different protein levels on branching morphogenesis of TAC-2 cells, experiments were carried out either in the presence or absence of sodium butyrate. We found that addition of sodium butyrate to the TAC-2 branching morphogenesis assay did not alter or enhance the TAC-2 cell phenotypes described below.

TAC-2 cells programmed to express LacZ give rise to small colonies with poorly branched cords when grown in collagen gels for 4 days (Fig. 2A). Addition of either HGF (20 ng/ml) or TGF-β2 (50 pg/ml) to the culture induces pronounced changes in colony morphology, resulting in the formation of long branching cords or tubules (Figs. 2B and 2C). We utilized TGF-β2 in our assays, which we found has an identical activity as TGF-β1 in the induction of branching morphogenesis of TAC-2 cells (Soriano *et al.,* 1996). When TAC-2 cells are programmed to express Wnt-1 proteins, cell colonies form cords with moderate branching even in the absence of exogenous growth factors (compare Figs. 2A and 2D). When TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (Figs. 2E and 2F), a highly extensive branching network is observed. This network of epithelial tubules is significantly more extensive when compared to control TAC-2 LacZ cells grown under identical conditions. An identical phenotype was observed in TAC-2 cell lines programmed to express either a non-epitope-tagged *Wnt*-1 cDNA or an HA epitope-tagged *Wnt*-1 cDNA transcribed from an SV40-based retroviral vector (data not shown). Thus, Wnt-1 activity was confirmed in at least three independently produced TAC-2 cell lines.

The morphological analysis of TAC-2 cell cultures suggested that Wnt-1 cooperates with either HGF or TGF-β2 in the induction of branching morphogenesis. To characterize combined effects of Wnt-1 and HGF, quantitative evaluation of cord length and the number of branch points was conducted (Figs. 3A and 3B). Both analyses showed that the branched network formed by TAC-2 Wnt-1 cells grown under control conditions was comparable to that found for HGF-treated TAC-2 LacZ cultures. When TAC-2 Wnt-1 cells are grown in the presence of HGF, both cord length and number of branch points is significantly greater than the combined values for TAC-2 Wnt-1 cells grown without HGF and TAC-2 LacZ cells grown with HGF (Figs. 3A and 3B). Thus, Wnt-1 and HGF act in a cooperative fashion to induce branching morphogenesis of TAC-2 cells.

To determine whether the effects of Wnt-1 on TAC-2 cell-branching morphogenesis are due to effects on the growth characteristics of TAC-2 cells, we compared the growth of the TAC-2 cell lines generated. TAC-2 cells were plated at different densities on collagen-coated dishes, in either the presence or absence of HGF, and viable cell numbers were determined either 2 or 6 days after plating. No significant differences in cell number were found between control TAC-2, TAC-2 LacZ, or TAC-2 Wnt-1 cell lines grown in either the presence or absence of HGF (data not shown). When grown under these conditions, TAC-2 Wnt-1 cells and control TAC-2 cells both displayed contact inhibition at confluence and had similar morphological characteristics. Hence, the effects of Wnt-1 on TAC-2 branching morphogenesis are not correlated with mitogenic activity of Wnt-1 and are dependent on growth in three-dimensional collagen gels. Identical results were obtained with TAC-2 cell lines that were programmed to express Wnt-1 using an SV40-based retroviral vector.

To further characterize morphogenetic activities of Wnt-1 proteins, we analyzed TAC-2 cells induced to form cyst structures in collagen gel cultures. When TAC-2 LacZ cells are grown in collagen gels in the presence of hydrocortisone and cholera toxin, they form spheroidal cysts enclosing a widely patent lumen, as previously observed with nontransfected TAC-2 cells (Soriano *et al.,* 1995) (Figs. 4A, 4C, and 4E). In contrast, under the same experimental conditions, TAC-2 Wnt-1 cells form branching structures (Figs. 4B, 4D, and 4F). This clearly indicates that Wnt-1 expression mod-

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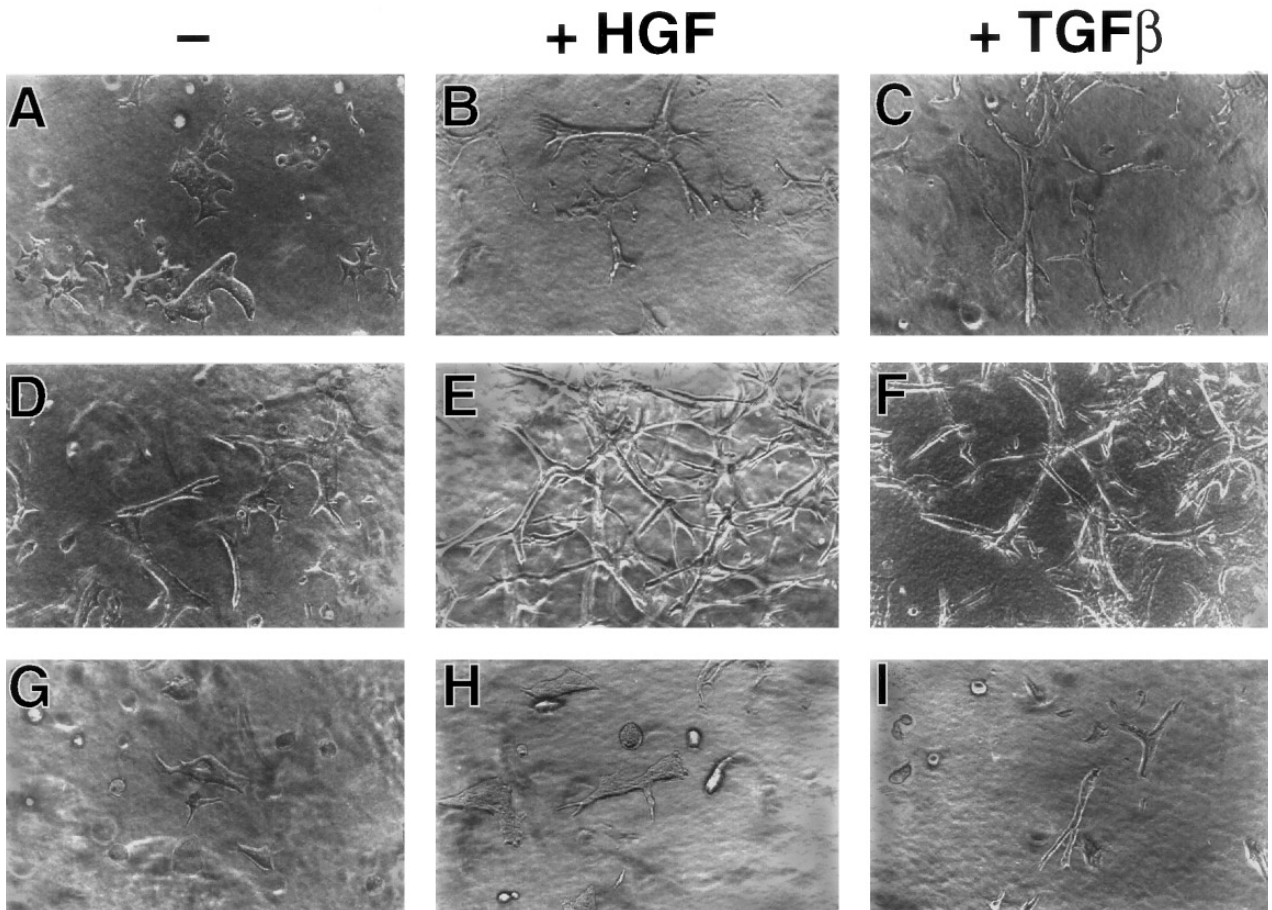

FIG. 2. TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ (A, B, C), Wnt-1HA (D, E, F), or int-3HA (G, H, I) were grown in collagen gels either in the absence of exogenous growth factor (A, D, G), in the presence of HGF (B, E, H), or TGF-β2 (C, F, I). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 cells in the absence of either HGF or TGF-β2 (compare D to A), and robust branching is observed when TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF-β2 (compare E to B, and F to C). TAC-2 cells programmed to express int-3 fail to undergo branching morphogenesis when grown in the presence of either HGF or TGF-β2 (compare G to H or I, H to B, and I to C).

ifies the spatial arrangement of TAC-2 cells and therefore has a morphogenetic effect.

Notch4(int-3) Inhibits TAC-2 Cell Branching Morphogenesis

Notch4 activity in branching morphogenesis was evaluated by expressing an activated form of the Notch4 receptor, Notch4(int-3), in TAC-2 cells. TAC-2 cell lines programmed to express the Notch4(int-3) proteins were generated using the retroviral vector pLNCX and will be referred to as TAC-2 int-3. Notch4(int-3) was HA-epitope tagged at the carboxy terminus to allow detection of ectopically expressed proteins. Immunoblot analysis using anti-HA antibodies detected Notch4(int-3) proteins that migrate with an approximate molecular weight of 60 kDa, corresponding well to their predicted molecular weight (Fig. 1).

When TAC-2 int-3 cells are grown in collagen gels and incubated in the presence of either HGF or TGF-β2 (Figs. 2H and 2I), cell colonies no longer form elongated cords like control cultures (Figs. 2B and 2C). Instead, HGF- or TGF-β2-treated TAC-2 int-3 cell colonies form small aggregates or structures with rudimentary branches which are similar in appearance to those formed by either TAC-2 LacZ or TAC-2 int-3 colonies grown in the absence of HGF or TGF-β2 (Figs. 2A and 2G). An identical phenotype was observed in at least three independently produced cell lines, including TAC-2 cells programmed to express a non-epitope-tagged *Notch4*(*int-3*) cDNA or a HA-epitope-tagged *Notch4*(*int-3*) cDNA transcribed from an SV40-based retroviral vector (data not shown). Interestingly, we found that a smaller percentage of TAC-2 int-3 cells give rise to colonies in collagen gels with respect to TAC-2 LacZ cells (230 $\pm$ 32 colonies/cm^2 in TAC-2 int-3 cells versus 795 $\pm$ 114

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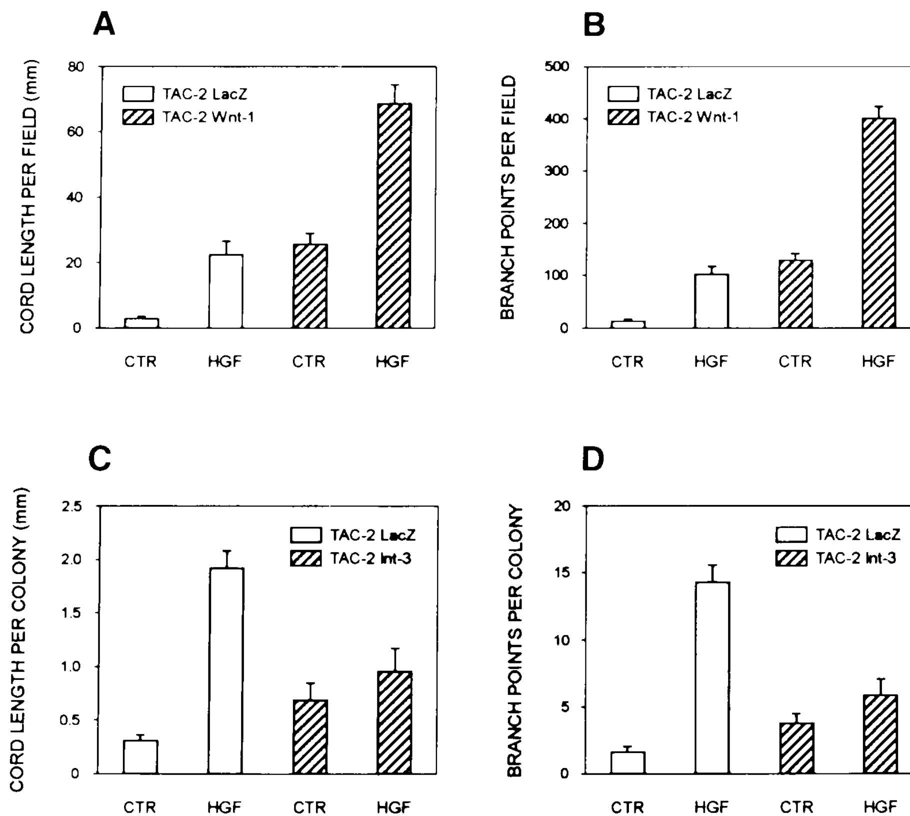

FIG. 3. Wnt-1 and HGF have cooperative effects on branching morphogenesis of TAC-2 cells, while expression of int-3 inhibits HGF-induced branching morphogenesis. TAC-2 LacZ, TAC-2 Wnt-1, and TAC-2 int-3 cells were suspended in collagen gels at 5×10^3 cells/ml (A and B) or 1×10^4 cells/ml (C and D) and incubated with either control medium or 10 ng/ml HGF for 7 days. In each of three separate experiments, at least three randomly selected fields per condition were photographed. The total additive length of all cords in each field (A), the number of cord branch points per field (B), the total additive length of all cords in each individual colony (C), and the number of cord branch points per colony (D) was determined as described under Materials and Methods. Values are means ± SEM, $n = 3$. Values for HGF are significantly ($P < 0.001$) different when compared to controls (except for TAC-2 int-3 cells) and values are significantly different ($P < 0.001$) when TAC-2 LacZ and TAC-2 Wnt-1 cell lines are compared. Similar results were obtained by evaluating cord length and branching per individual TAC-2 LacZ and TAC-2 Wnt-1 colony (data not shown).

colonies/cm^2 in TAC-2 LacZ cells), which suggests that Notch4(int-3) expression reduces plating (colony formation) efficiency in collagen gels. Accordingly, to avoid overestimating the inhibition of HGF-induced cord elongation in TAC-2 int-3 cells, the quantitative analysis of cord length and branching was carried out on a per colony basis, rather than on a per field basis (see Materials and Methods). This analysis demonstrated that, despite the fact that colonies formed by TAC-2 int-3 cells are slightly more elongated and branched than those formed by TAC-2 LacZ cells, their morphogenetic response to HGF is markedly decreased (Figs. 3C and 3D).

We analyzed the growth characteristics of the TAC-2 int-3 cell line, as described for TAC-2 Wnt-1 cells. TAC-2 int-3 cells plated on collagen-coated dishes, in either the presence or absence of HGF, displayed no significant differences in cell number, morphology, or growth postconfluence when compared with TAC-2 controls (data not shown). Identical results were obtained with TAC-2 cell lines programmed to express Notch4(int-3) using an SV40-based retroviral vector. Hence, the effects of Notch4(int-3) on TAC-2 branching morphogenesis are not correlated to changes in the growth properties in the cells.

The Carboxy Terminus of the Notch4(int-3) Is Not Required for Activity

The Notch4(int-3) oncoprotein has most of the extracellular domain of Notch4 deleted and consists of the transmembrane and intracellular domains. To investigate which region(s) of Notch4(int-3) proteins are required and sufficient for activity, a series of Notch4(int-3) deletion mutants were generated (schematized in Fig. 5A). Four Notch4(int-3) deletion mutants were made and designated ΔNT (deletion of the amino terminal domain), ΔCDC (deletion of cdc10 repeat domain), ΔCT (deletion of the carboxy terminal do-

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main), and ΔNTΔCT (N-terminal and C-terminal deletion) (Fig. 5A). All four mutant int-3 cDNAs were HA-epitope tagged at their carboxy termini and TAC-2 cell lines programmed to express each deletion mutant were generated using the retroviral vector pLNCX. Immunoblot analysis using anti-HA monoclonal antibodies demonstrated expression of Notch4(int-3) deletion proteins of appropriate molecular weight in each respective cell line (Fig. 5B). The ΔNTΔCT Notch4(int-3) deletion protein with predicted molecular weight of 25 kDa comigrates with a nonspecific anti-HA antibody background band (Fig. 5B); however, it was detected in a separate analysis of both untreated TAC-2 ΔNTΔCT cells or cells treated with sodium butyrate (Fig. 5C). TAC-2 cell lines expressing the four different Notch4(int-3) deletion mutants were grown in collagen gels as described above, and the ability of each Notch4(int-3) deletion mutant to inhibit HGF-induced branching morphogenesis of TAC-2 cells was analyzed. As shown in Fig. 6, TAC-2 cells expressing either ΔNT (Figs. 6A and 6B), ΔCDC (Figs. 6C and 6D), or ΔNTΔCT (Figs. 6G and 6H) are responsive to HGF-induced branching morphogenesis.

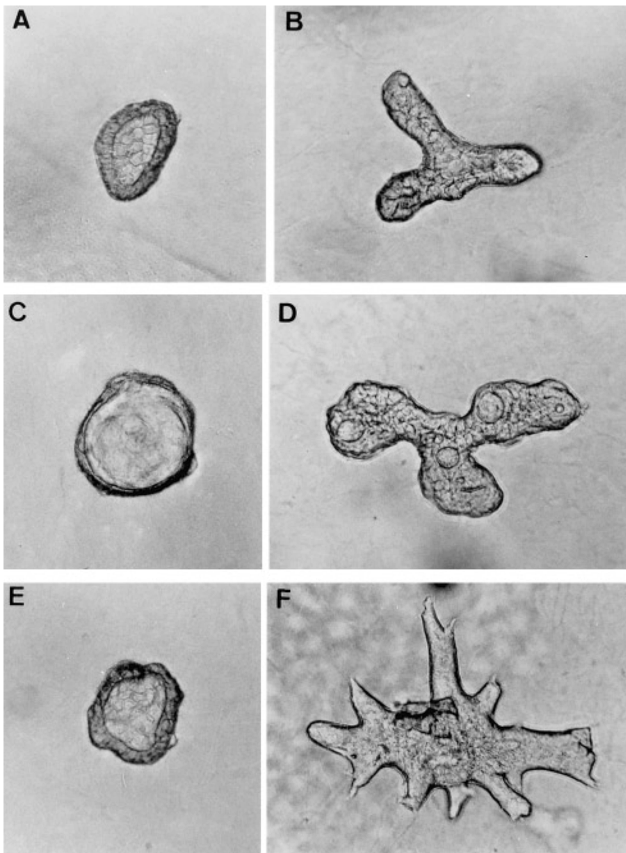

FIG. 4. Differential behavior of TAC-2 LacZ cells and TAC-2 Wnt-1 cells in hydrocortisone-supplemented cultures. Cells were suspended in collagen gels at 5×10^3 cells/ml and incubated for 10 days with 1 μg/ml hydrocortisone and 50 ng/ml cholera toxin. Under these conditions, TAC-2 LacZ cells form thick-walled spheroidal cysts enclosing a widely patent lumen (A, C, E), as previously shown for untransfected cells. In marked contrast, TAC-2 Wnt-1 cells form branched structures consisting of short tubules (B), cords containing small multifocal lumina (D), or apparently solid cords (F). The three-dimensional structures illustrated in A, C, E and B, D, F are representative of the vast majority of colonies formed by TAC-2 LacZ and TAC-2 Wnt-1 cells, respectively. Original magnification, 180×.

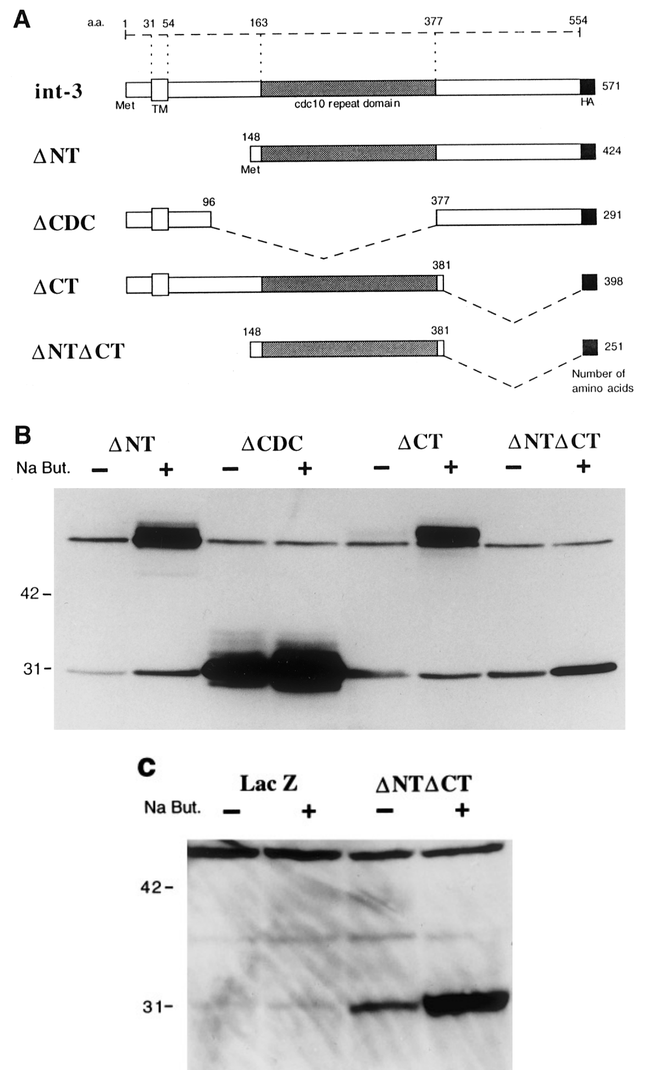

FIG. 5. Schematic representation of int-3 deletion mutants (A) and immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies (B, C). TAC-2 cells programmed to express ΔNT, ΔCDC, ΔCT, and ΔNTΔCT were grown in the presence or absence of sodium butyrate. The int-3 deletion proteins are epitope tagged and deletion proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment. (C) Immunoblot analysis on lysates of TAC-2 LacZ and TAC-2 ΔNTΔCT cells, as in B, demonstrating the presence of the ΔNTΔCT deletion protein.

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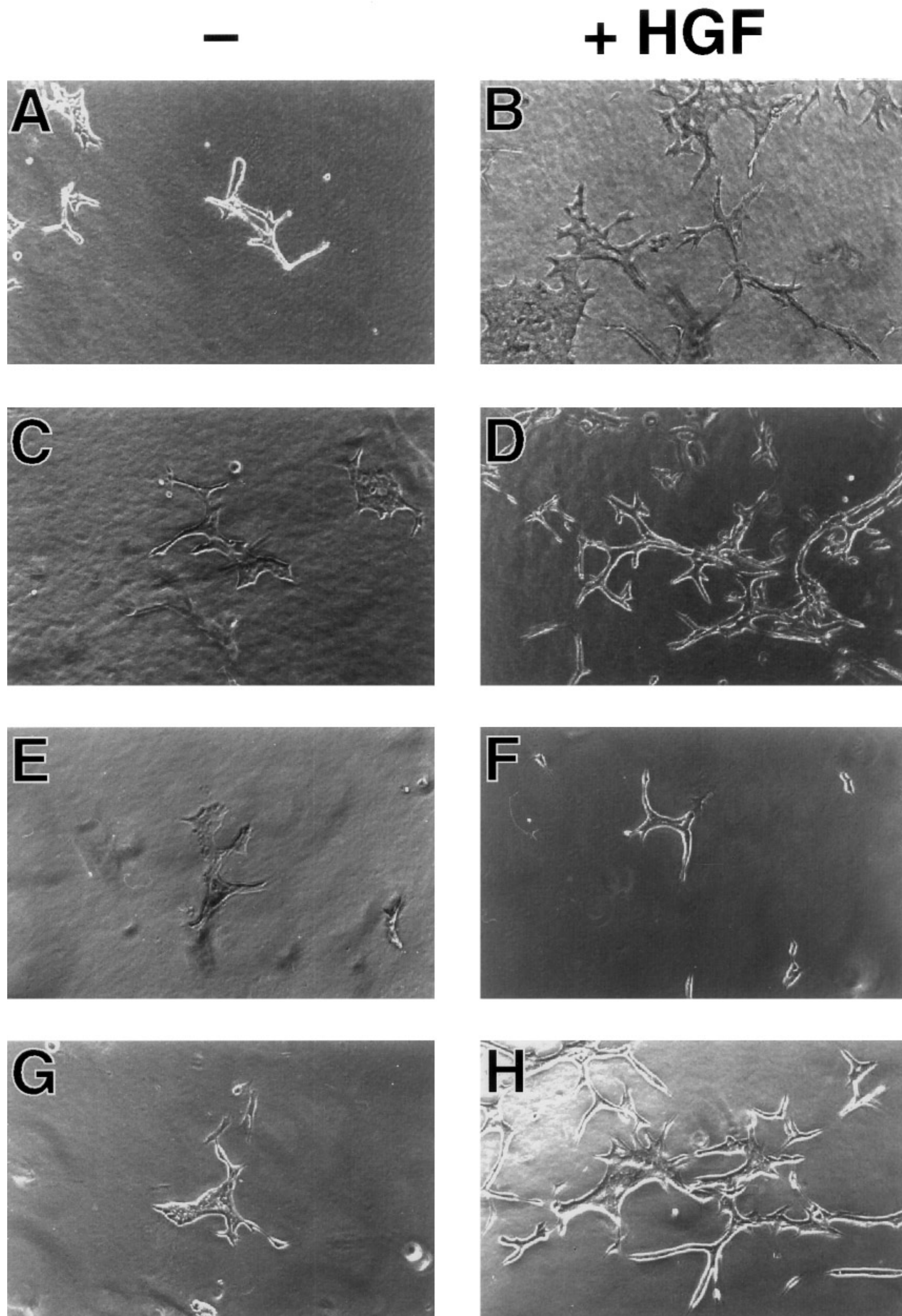

FIG. 6. TAC-2 cell ductal morphogenesis assay with int-3 mutants. TAC-2 cells programmed to express ΔNT (A, B), ΔCDC (C, D), ΔCT (E, F), and ΔNTΔCT (G, H) were grown in collagen gels either in the absence of exogenous growth factor (A, C, E, G) or in the presence of HGF (B, D, F, H). HGF induces branching morphogenesis of TAC-2 ΔNT cells (B), TAC-2 ΔCDC cells (D), and TAC-2 ΔNTΔCT cells (H). TAC-2 ΔCT cells fail to undergo branching morphogenesis when grown in the presence of HGF (F).

In contrast, when grown in the presence of HGF, ΔCT expressing TAC-2 cells (Figs. 6E and 6F) display an identical phenotype as the TAC-2 int-3 cells. Hence, the carboxy terminus of the Notch4(int-3) is not required for Notch-mediated inhibition of TAC-2 branching morphogenesis. Thus, in this assay the activity of the Notch4(int-3) oncoprotein can be conferred by the amino terminus and cdc10 repeats.

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Branching Morphogenesis in Cells Coexpressing Wnt-1 and Notch4(int-3) Oncoproteins

The activation of the Wnt-1 and Notch signaling pathways resulted in opposite effects on HGF- or TGF-β2-induced branching morphogenesis of TAC-2 cells. To explore the interactions between these two signaling pathways, we investigated the effect of simultaneous expression of both Wnt-1 and Notch4(int-3) proteins on TAC-2 branching morphogenesis. The above-described TAC-2 LacZ and TAC-2 int-3 cell lines, which were generated with the pLNCX expression vector, were now also programmed to express Wnt-1 using the retroviral vector pLHTCX. This vector drives gene expression from the CMV promoter and contains the hygromycin resistance gene. In this fashion, four additional TAC-2 cell lines were generated that were named TAC-2 LacZ/ctr, TAC-2 LacZ/Wnt-1, TAC-2 int-3/ctr, and TAC-2 int-3/Wnt-1 (where ctr denotes control empty pLHTCX vector). To determine appropriate protein expression in each of these four cell lines, immunoblot analysis showed Notch4(int-3) and Wnt-1 proteins were expressed as expected and at levels similar to those previously found to confer activity (data not shown). Each of the four cell lines were grown in collagen gels to determine their ability to undergo HGF- or TGF-β2-induced branching morphogenesis (Fig. 7). This assay was repeated three times with similar results. Doubly infected control cells TAC-2 LacZ/ctr (Figs. 7A–7C) remained responsive to both HGF and TGF-β, demonstrating that two rounds of drug selection did not affect the phenotype of the TAC-2 cell lines. As observed previously for TAC-2 Wnt-1 cells, TAC-2 LacZ cells that are now programmed to express Wnt-1 (Figs. 7D–7F) form small colonies that undergo modest branching even in the absence of HGF or TGF-β2; these cells form extensive elongated branches when grown in the presence of HGF or TGF-β2. The activity found for Notch4(int-3), that is the inhibition of HGF- and TGF-β-induced branching morphogenesis, was also found in the TAC-2 int-3/ctr cell line (Figs. 7G–7I). Wnt-1- and Notch4(int-3)-coexpressing cells, TAC-2 int-3/Wnt-1, are able to form colonies displaying branching and elongation and have an appearance similar to that of TAC-2/LacZ/Wnt-1 cells (Fig. 7J). An examination of several fields reveals that TAC-2 int-3/Wnt-1 cells displayed increased responses when treated with either HGF or TGF-β, thus these cells now regain responsiveness to these factors (Figs. 7K and 7L). Our results indicate that Notch activation attenuates responsiveness of TAC-2 cells to both HGF and TGF-β and that Wnt-1 can override the Notch activity in TAC-2 cells.

DISCUSSION

In this study, we have detailed a regulatory hierarchy involved in the branching morphogenesis of TAC-2 mammary epithelial cells. This regulation includes four distinct signaling pathways: the Wnt, Notch, HGF, and TGF-β signaling cascades. Using an *in vitro* model that reflects the branching morphogenesis exhibited during mammary gland development, we have assessed the potential interactions between several different signaling pathways. This approach has allowed us to establish the relationships between these pathways. One remarkable feature of the regulation of branching morphogenesis we describe is its similarity to the regulatory pathways leading to morphogenetic events during *Drosophila* development.

Wnt Proteins as Branching Morphogens in the Mammary Gland

Formation of branching cords is induced in collagen gel cultures of TAC-2 cells by the addition of either HGF or low concentrations of TGF-β. Wnt-1 proteins induce moderate branching and elongation of TAC-2 cell tubules in the absence of added HGF or TGF-β2. The extent of Wnt-1-induced branching morphogenesis of TAC-2 cells is comparable to the induction by either HGF or TGF-β2. Our evidence suggests that Wnt-1 acts as a morphogen in this capacity. First, Wnt-1 induces a change in a morphogenetic event, the formation of branched epithelial tubules or cords. Second, Wnt-1 does not appear on its own to alter the growth properties of the TAC-2 cells. Finally, Wnt-1 can induce branching in an environment where cysts typically form, that is, in hydrocortisone- and cholera toxin-treated cultures. Such cultures form spheroidal cysts enclosing a widely patent lumen. In this environment, peptide growth factors that display mitogenic and not morphogenetic properties would increase the size of the cyst but the spheroidal structure would be maintained. In contrast, Wnt-1 alters the morphogenetic behavior in such a way that new branch points are formed and the structures take on a tubular morphology.

Several Wnt proteins are expressed in the mammary gland during periods of morphological changes of the ductal epithelium (*Wnt*-2, *Wnt*-5a, *Wnt*-7, and *Wnt*-10b) (Gavin and McMahon, 1992; Weber-Hall *et al.,* 1994). Ectopic expression of Wnt-1 *in vivo* suggests a role for Wnt proteins in cell proliferation during mammary gland development; however, morphogenetic changes also occur in response to Wnt-1. It is has been proposed that the Wnt-1 expression mimics the activity of endogenous mammary gland Wnt proteins. A transgenic line driving expression of *Wnt*-1 to the mammary gland displays a hyperplastic phenotype, indicative of increased proliferation (Tsukamoto *et al.,* 1988). In addition, both virgin females and males display a marked increase in the number of terminal branches, and in fact resemble the hormonally stimulated glands normally observed in pregnant animals. Tissue reconstitution experiments in which *Wnt*-1 is ectopically expressed in mammary epithelium also result in a hyperplastic gland where duct epithelium show abundant fine side-branches, suggesting that Wnt-1 may instruct the epithelium to form branches (Edwards *et al.,* 1992). This phenotype is most likely not simply a consequence of proliferation as it is not seen with a variety of other oncogenic proteins which when ectopically

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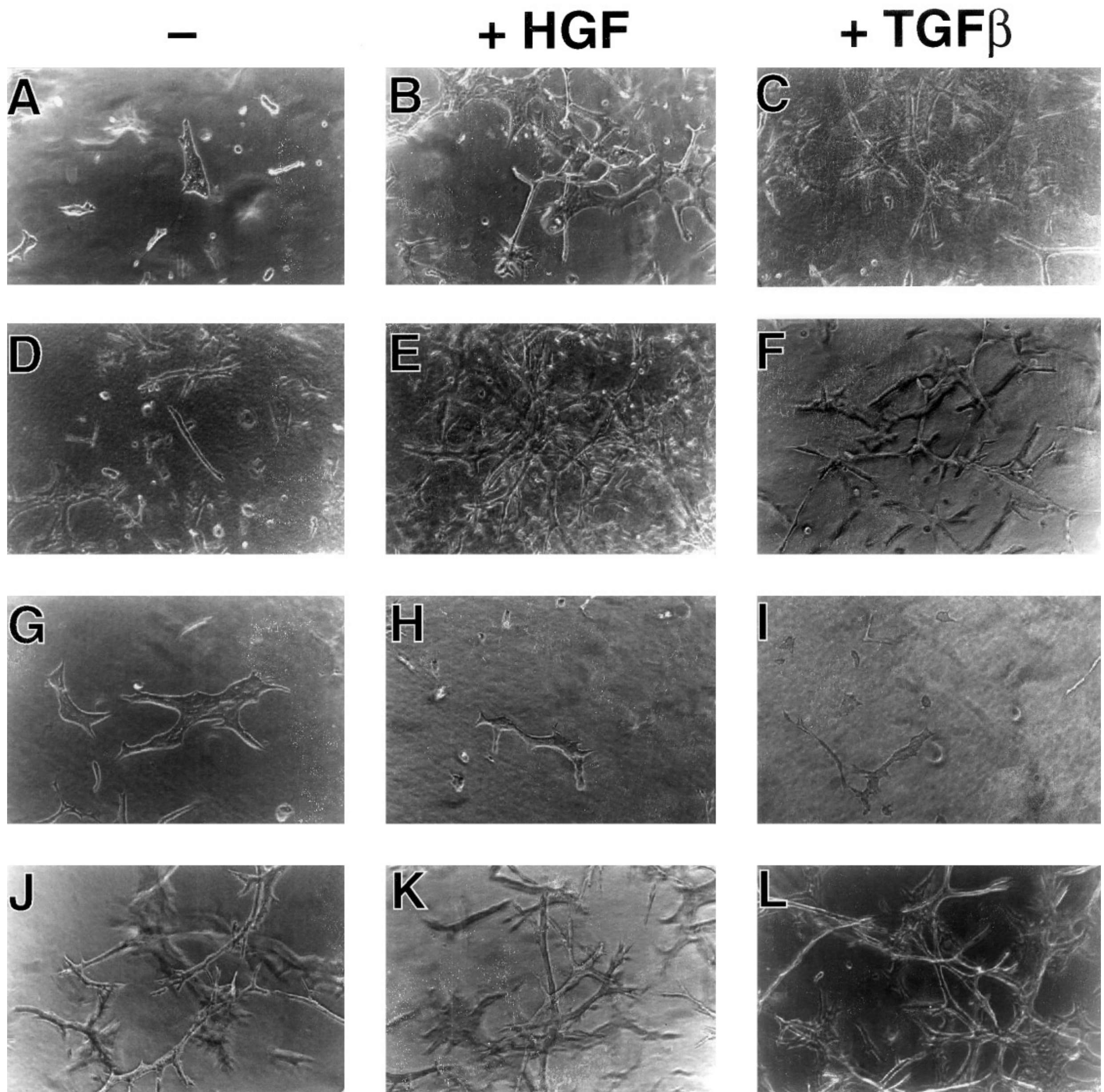

FIG. 7. TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ/ctr (A, B, C), LacZ/Wnt-1 (D, E, F), int-3/ctr (G, H, I), or int-3/Wnt-1 (J, K, L) were grown in collagen gels in the absence of exogenous growth factor (A, D, G, J), in the presence of HGF (20 ng/ml) (B, E, H, K), or in the presence of TGF-β2 (50 pg/ml) (C, F, I, L). HGF and TGF-β2 induce branching morphogenesis of TAC-2 LacZ/ctr cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 LacZ/Wnt-1 cells in the absence of either HGF or TGF-β2 (D), and robust branching is observed when TAC-2 LacZ/Wnt-1 cells are grown in the presence of either HGF (E) or TGF-β2 (F). TAC-2 cells programmed to express int-3/ctr fail to undergo branching morphogenesis when grown in the presence of either HGF (H) or TGF-β2 (I). TAC-2 cells programmed to express both int-3 and Wnt-1 undergo branching morphogenesis in the absence of exogenous growth factor (J), and form a robust branching network when grown in the presence of HGF (K) or TGF-β2 (L) in a similar manner when compared to TAC-2 cells programmed to express Wnt-1 solely (D, E, F).

expressed in the mammary gland induce hyperplasia without increasing branching (Edwards, 1993).

The Wnt signal transduction pathway is mediated in part through β-catenin, a protein associated with cadherins, and which is necessary for the adhesive functions of adherens junctions (Miller and Moon, 1996). Wnt-1 signaling results in stabilization of the cytoplasmic pool of β-catenin (Lin *et al.,* 1997; Shimizu *et al.,* 1997), which then can associate

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with downstream targets in the cytoplasm to transduce signals that lead to regulation of target gene expression (Molenaar *et al.,* 1996; Morin *et al.,* 1997). TAC-2 cells programmed to express Wnt-1 in fact display increased levels of cytosolic β-catenin, when compared to TAC-2 LacZ cells (our unpublished data). This stabilization may be regulated by the phosphorylation of β-catenin on serine/threonine residues, possibly by glycogen synthase kinase 3 (GSK-3) (Yost *et al.,* 1996). Recent evidence has demonstrated the importance of β-catenin/cadherin interactions in regulating cell adhesion, cell migration, and epithelial phenotype in embryonic development (Gumbiner, 1996). The activation of β-catenin by Wnt-1-induced signaling may result in changes of the adhesive and migratory characteristics of mammary epithelial cells and consequently affect ductal morphogenesis of TAC-2 cells.

Cooperative Interactions between Wnt-1 and HGF or TGF-β

In response to the combined effects of Wnt-1 and HGF, TAC-2 cells form a network of elongated and branching tubules that is far more extensive than the branching cords observed when TAC-2 cells are grown in the presence of Wnt-1 or HGF singularly. We propose that the combined effect of HGF addition and Wnt-1 expression is not the result of the sum of their independent activities on branching morphogenesis, but that Wnt signaling synergizes with the HGF/c-met tyrosine kinase pathway. The possibility that Wnt proteins cooperate *in vivo* with the HGF/c-met pathway in the regulation of mammary morphogenesis is supported by the overlapping temporal patterns of *Wnt* genes and HGF/c-met expression (Gavin and McMahon, 1992; Pepper *et al.,* 1995; Weber-Hall *et al.,* 1994).

One potential area where these two signaling pathways could converge might be through their effects on the catenin and cadherin proteins. The cooperation between Wnt-1 and HGF may be explained by their combined activation of β-catenin. β-Catenin has been detected in a complex with the EGF receptor and can be phosphorylated in response to EGF and HGF (Hoschuetzky *et al.,* 1994; Shibamoto *et al.,* 1994). In addition, the Ras pathway is essential for the biological activity induced by HGF/c-met (Hartmann *et al.,* 1994) and β-catenin has been demonstrated to be a substrate for tyrosine kinases and to become tyrosine phosphorylated in cells expressing activated Src and Ras (Behrens *et al.,* 1993). Another catenin-like protein, p120, which was identified as a substrate of Src and several receptor tyrosine kinases, interacts with the cadherin–β-catenin complex and may participate in regulating the adhesive function of cadherins (Daniel and Reynolds, 1995). EGF is also able to stimulate branching morphogenesis of TAC-2 cells, although not to the same extent as HGF, whereas NGF, bFGF, IGF-II, and KGF cannot (Soriano *et al.,* 1995). These activities correlate with the reported phosphorylation of β-catenin by the EGF and HGF signal transduction pathways (Shibamoto *et al.,* 1994). It is yet unclear how tyrosine phosphorylation of β-catenin might regulate its activity. Tyrosine phosphorylated β-catenin is found in a detergent soluble pool (Hoschuetzky *et al.,* 1994; Kinch *et al.,* 1995), which may reflect specific phosphorylation of a free pool of β-catenin. Since both Wnt-1 and HGF signaling can converge on β-catenin, it is therefore possible that the observed cooperation between HGF and Wnt-1 is due to their combined action on β-catenin activity.

Wnt-1, HGF, and TGF-β could induce branching morphogenesis by regulating the adhesive and migratory properties of TAC-2 cells through modulation of extracellular matrix components and their interaction with their receptors. Since HGF has been demonstrated to decrease adhesion of TAC-2 cells to collagen and to enhance the deposition of type IV collagen (Soriano *et al.,* 1995), it is also possible that the observed cooperation between HGF and Wnt-1 is due to their combined effect on cell–substrate adhesion. TGF-β signaling involves receptors with serine/threonine kinase activity which are known to regulate the synthesis and degradation of extracellular matrix molecules and to induce matrix organization. Induction of branching morphogenesis by TGF-β could be mediated by a remodeling of extracellular matrix components and cell–substrate interactions. The Wnt signal transduction pathway may also regulate cell–substrate interactions, and the combined activity of both Wnt-1 and TGF-β may explain their cooperative activities on the branching morphogenesis of TAC-2 cells.

In *Drosophila,* wingless (*wg*) and the TGF-β homologue *Decapentaplegic* (*Dpp*) have been shown in some cases to act in combination to regulate gene transcription during inductive events. In particular, Wg and Dpp have been shown to act in combination during limb development (Campbell *et al.,* 1993; Diaz-Benjumea *et al.,* 1994) and to induce *Ultrabithorax* expression during endoderm induction (Riese *et al.,* 1997). Wnt-1 and TGF-β signaling may similarly converge to affect gene transcription during branching morphogenesis in the mouse mammary gland.

Notch Inhibits Branching Morphogenesis of Mammary Epithelial Cells

We demonstrate that Notch activation inhibits both the HGF- and TGF-β-induced branching morphogenesis of TAC-2 mammary epithelial cells. The precise mechanism of this inhibition is unclear. Activation of Notch signaling has been demonstrated to inhibit or alter the cell fate commitment or differentiation of a variety of different cell types (Artavanis-Tsakonas *et al.,* 1995; Greenwald and Rubin, 1992). For instance, *C. elegans* Lin-12 controls cell fate decisions during gonadogenesis, *Drosophila Notch* acts to control cell fate during neuroblast and photoreceptor cell differentiation, an activated *Xenopus Notch* can affect epidermal and neural crest cell development, and an activated mouse *Notch*1 can control cell fate during myogenesis and neurogenesis of cultured mouse cells (Artavanis-Tsakonas *et al.,* 1995; Greenwald and Rubin, 1992). Transgenic mice that use the MMTV viral promoter to express the Notch4(int-3) oncoprotein, the activated form of Notch4, display severely impaired mammary ductal growth (Jhappan *et al.,* 1992).

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When *Notch4*(*int-3*) is expressed from the whey acidic protein promoter, whose expression is restricted to the secretory mammary epithelial cells, the differentiation of the secretory lobules of the transgenic animals is profoundly inhibited (Gallahan *et al.*, 1996). These experiments demonstrate that *Notch4*(*int-3*), like many other activated *Notch* genes, can act as a regulator of cell fate decisions in the mammary gland of mice. Little is known about the spatial and temporal pattern of *Notch* gene expression in the mammary gland; however, *Notch4* is expressed *in vivo* in the murine mammary gland (Sarkar *et al.*, 1994; Smith *et al.*, 1995). *Notch* genes may thus regulate the cell fate decisions occurring during mammary gland development that lead to the branched epithelial structure of the gland.

We have found that Notch activation can affect the response of TAC-2 mammary epithelial cells to either HGF or TGF-β. Since HGF acts through a tyrosine kinase receptor and TGF-β acts through serine/threonine kinase receptors, the effects of Notch activation may involve more than specific inhibition of a particular signaling cascade. Notch may regulate the competency of TAC-2 cells to respond to several different factors, possibly by shifting TAC-2 cells to a fate that is not predisposed to undergo branching morphogenesis. This model would be consistent with the proposed activities of Notch proteins in several different organisms. Alternately, these signaling pathways may be controlled by Notch at a point at which they may converge to induce expression of genes important for branching morphogenesis. Recently, the intracellular domain of LIN-12, a *C. elegans* Notch, has been demonstrated to associate with EMB-5, which encodes for a cytoplasmic protein containing a SH2 domain (Hubbard *et al.*, 1996). This finding raises the possibility that the Notch-signaling proteins may interact directly with those elicited by tyrosine kinase receptors, such as the HGF receptor (c-met).

We have demonstrated that the domain, carboxy terminal to the cdc10 repeats, of the Notch4(int-3) oncoprotein is not required for biological activity. However, the amino terminal domain and the cdc10 repeats are required for Notch4(int-3) activity. These findings are consistent with previous observed data for other *Notch* genes. The RAM23 domain which is localized between the transmembrane and cdc10 repeats has been demonstrated to be the binding site of CBF-1, a downstream and essential element in Notch signaling (Hsieh *et al.*, 1996). Deletion of the amino terminal domain of Notch4(int-3), which contains the RAM23 domain, may eliminate binding to CBF-1, and hence destroy Notch4(int-3) activity. The region of the LIN-12 protein that includes the RAM-23 domain and cdc10 repeats appears to interact with another downstream and positive regulator, EMB5 (Hubbard *et al.*, 1996). Point mutations and deletions within the cdc10 repeats result in loss of function of Notch proteins (Greenwald, 1994). Our data thus indicate that Notch4 may interact and be regulated through similar mechanisms.

Competing Influences of Wnt and Notch Signaling in Branching Morphogenesis

When TAC-2 cells are programmed to express both Wnt-1 and Notch4(int-3), the cells are able to undergo branching morphogenesis. In Wnt-1- and Notch4(int-3)-coexpressing TAC-2 cells, branching morphogenesis can be increased by either HGF or TGF-β; that is, the cells regain responsiveness to these factors. The phenotype observed in Wnt-1- and Notch4(int-3)-coexpressing cells was similar to that of TAC-2 cells expressing only Wnt-1. The opposite biological activities of Wnt-1 and Notch4(int-3) observed in the TAC-2 cell assay correlate well with the mammary gland phenotype observed in Wnt-1 and Notch4(int-3) transgenic mice that ectopically express these proteins in the mammary gland (Jhappan *et al.*, 1992). Although both oncogenes increase mammary tumor development, Wnt-1 stimulates a hyperplastic phenotype with increased ductal development, whereas Notch4(int-3) inhibits ductal development.

Wnt-1 can override the Notch4(int-3)-mediated inhibition of branching morphogenesis providing the first evidence of interaction between these two signaling pathways in vertebrates. The dominance of Wnt-1 over activated Notch which we have observed in murine cells parallels the functional relationship proposed for *Drosophila Wnt* (*wingless*) and *Notch* during *Drosophila* development (Axelrod *et al.*, 1996). In this study, genetic analysis suggests a pathway convergence between wingless and Notch signaling, resulting in opposing effects during patterning of the developing *Drosophila* wing. Activation of the wingless signal leads to regulation of Notch activity, possibly by *Drosophila* dishevelled, a cytoplasmic protein that is also a positive mediator in the Wnt-1 signal transduction pathway. Analysis using a yeast interaction trap system demonstrated that Dishevelled physically associates with the intracellular domain of Notch. The antagonism between Wnt-1 and Notch4(int-3) seen in branching morphogenesis may also be mediated by common regulators of the two signaling pathways such as Dishevelled.

During mammary gland development, the growth and differentiation of the gland is regulated by mesenchymal–epithelial and epithelial–epithelial interactions. Cells often receive different signals simultaneously and must integrate them in order to take on the correct proliferative, morphogenetic, or differentiative response. Notch inhibition of ductal morphogenesis may be an early event in ductal morphogenesis. An attractive mechanism for overcoming Notch and allowing ductal morphogenesis to initiate or progress would be to activate the expression of a *Wnt* gene(s). Wnt could then serve the dual function of suppressing Notch activity and initiating branching morphogenesis. Wnt signaling may then cooperate with other signaling pathways, such as those mediated by HGF and TGF-β, in order to complete branching morphogenesis. Our study thus has revealed complex interactions between the signal transduction pathways of Wnt, Notch, HGF, and TGF-β, in regulating the branching morphogenesis of mammary epithelial cells.

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