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Advances in Brief

Expression of *cripto*, a Novel Gene of the Epidermal Growth Factor Gene Family, Leads to *in Vitro* Transformation of a Normal Mouse Mammary Epithelial Cell Line

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Abstract

cripto **is a gene encoding an epidermal growth factor-related protein that is expressed in undifferentiated embryonal carcinoma cells. To ascertain if *cripto* is capable of functioning as a transforming gene, a full-length human *cripto* complementary DNA under the transcriptional control of the Rous sarcoma virus long terminal repeat has been cotransfected with the selectable pSV2*neo* marker plasmid into immortalized mouse NOG-8 mammary epithelial cells. Several neomycin-resistant clones were isolated that express high levels of a specific *cripto* 4.5-kilobase mRNA transcript and possess multiple copies of *cripto* plasmid DNA. NOG-8 cells that express *cripto* are able to clone in soft agar and exhibit an approximately 3-fold increase in their anchorage-dependent growth in serum-free medium as compared to the *neo*-transfected NOG-8 cells. However, none of the *cripto*-expressing NOG-8 clones are able to form tumors in nude mice.**

Introduction

cripto is a gene recently identified and cloned from a human embryonal carcinoma cell line, NTERA2 clone D1 (NT2D1) (1). A specific 2.2-kilobase *cripto* mRNA is expressed in undifferentiated NT2D1 cells but not in differentiated NT2D1 cells. The human *cripto* cDNA[2] is 2020 base pairs long with an open reading frame of 564 base pairs, a 245-base pair-long 5′-untranslated region, and a 1209-base pair-long 3′-untranslated region containing an inverted Alu repeat. The open reading frame encodes for a protein of 188 amino acids that contains a central portion of approximately 37 amino acids which shares a structural cysteine-rich motif in common with other members of the EGF supergene family, such as human EGF, human TGF α, and human amphiregulin (1, 2). The human *cripto* gene maps to chromosome 3, is approximately 7 kilobases long, and contains 6 exons and 5 introns.[3]

It has been demonstrated previously that overexpression of EGF or TGF α cDNAs in fibroblasts and epithelial cells can lead to transformation *in vitro* and in certain cases to tumorigenicity *in vivo* (3–9). NOG-8 cells are an EGF-responsive, spontaneously immortalized, nontumorigenic mammary epithelial cell line (10). NOG-8 cells can be transformed following transfection or infection with expression vectors containing a single activated oncogene, such as the human point-mutated c-Ha-*ras* or the rat point-mutated c-*neu* (c-*erbB*-2) protooncogenes (10–12). Similarly, overexpression of a human TGF α cDNA is sufficient to transform NOG-8 cells (7, 8).

Preliminary experiments have shown that transfection of a human *cripto* cDNA expression vector in which transcription is under the control of the RSV LTR into mouse NIH-3T3 fibroblasts induces the formation of transformed foci (1). Since *cripto* and TGF α are expressed in undifferentiated human embryonal carcinoma cells in which they may function as autocrine growth factors (1, 13) and since a portion of the predicted *cripto* protein shares significant homology to EGF and to TGF α, we have transfected NOG-8 mouse mammary epithelial cells with an RSV LTR-*cripto* expression vector plasmid to ascertain whether *cripto* may function as a transforming gene in epithelial cells.

Materials and Methods

Cell Cultures and Transfection. NOG-8 cells, a spontaneously immortalized normal mouse mammary epithelial cell line (10), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing 4 mM glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), streptomycin (100 μg/ml), and penicillin (100 units/ml) (GIBCO, Grand Island, NY) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. NOG-8 *cripto* cells were generated by cotransfection of NOG-8 cells with an expression vector plasmid containing the human *cripto* cDNA under the transcriptional control of the RSV LTR (1) and with the pSV2*neo* plasmid. Transfection by the calcium phosphate precipitation method was performed as described previously (7). Following 21 days of culture in selective medium containing geneticin (G418; GIBCO), 800 μg/ml, 4 of 22 individual G418-resistant NOG-8 *cripto* clones were randomly selected and expanded into cell lines. NOG-8 *neo* cells are NOG-8 cells that have been transfected with the pSV2*neo* expression vector plasmid alone. NOG-8 *ras* cells are NOG-8 cells that have been transformed following cotransfection of an expression vector plasmid containing the activated point-mutated human c-Ha-*ras* protooncogene under the transcriptional control of the mouse mammary tumor virus LTR and of the pSV2*neo* plasmid, as described previously (11).

Monolayer Growth. Two × 10^4 cells/well were plated in 12-multiwell cluster dishes (Costar, Cambridge, MA). Twenty-four h later, the cells were washed twice, incubated in PC-1 serum-free medium (Ventrex, Portland, ME) for 4 days, trypsinized, and counted with a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL).

Soft Agar Growth. Two × 10^4 cells were suspended in 1 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and layered over 1 ml of an 0.8% agar-medium base layer in 35-mm dishes (Costar). After 18 days, the cells were stained with nitroblue tetrazolium

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[2] The abbreviations used are: cDNA, complementary DNA; EGF, epidermal growth factor; TGF α, transforming growth factor α; RSV, Rous sarcoma virus; LTR, long terminal repeat; poly(A)$^+$ RNA, polyadenylated RNA.

[3] R. Dono, N. Montuori, M. Rocchi, L. De Ponti-Zilli, A. Ciccodicola, and M. G. Persico. Structure and genomic location of two members of the *cripto* gene family, submitted for publication.

and counted with an Artek 880 colony counter (Artek Systems, Farmingdale, NY).

DNA Isolation and Southern Blot Analysis. High molecular weight DNA was extracted from the cells using sodium dodecyl sulfate-proteinase K, as described previously (7). DNA (10 μg) was digested with the *Eco*RI restriction endonuclease before being fractionated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled nick-translated 900-base pair *Eco*RI-*Eco*RI human *cripto* cDNA insert (1).[3]

RNA Isolation and Northern Blot Analysis. Total cellular RNA was extracted by lysis of the cells in guanidine thiocyanate and centrifugation over a cesium chloride cushion (14). Poly(A)$^+$ RNA was obtained by absorption to and elution from an oligodeoxythymidylate cellulose column (type III; Collaborative Research, Lexington, MA). Ten μg of poly(A)$^+$ RNA were electrophoresed through a denaturating 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 transferred to Biotrans nylon membranes (ICN Biomedicals, Costa Mesa, CA) and hybridized to the following ^{32}P-labeled nick-translated cDNA probes: a 900-base pair *Eco*RI-*Eco*RI human *cripto* cDNA insert (1)[3]; and a 770-base pair human β-actin cDNA insert (Oncor, Gaithersburg, MD).

Tumorigenicity in Nude Mice. Groups of 10 female BALB/c nude (*nu*$^+$/*nu*$^+$) mice were given s.c. injections into the dorsal flank of 5 × 10^6 NOG-8 cells, NOG-8 *neo* cells, NOG-8 *ras* cells, or different NOG-8 *cripto* clones, as described previously (7). The animals were monitored for the appearance of tumors for a 4-month period.

Results and Discussion

cripto has a conserved cysteine-rich region that may be involved in the formation of three disulfide bonds in a structure analogous to those of EGF, TGF α, and amphiregulin (1, 2). To determine whether *cripto* is also functionally related to EGF and if it may act as a transforming gene in epithelial cells, we have introduced an expression vector plasmid containing the human *cripto* cDNA under the transcriptional control of the RSV LTR into NOG-8 cells. NOG-8 cells were selected since they are an EGF-responsive, spontaneously immortalized, near diploid, nontransformed mouse mammary epithelial cell line (10, 11). Moreover, NOG-8 cells can be transformed *in vitro* and *in vivo* following introduction of a recombinant expression vector containing the human TGF α cDNA under the transcriptional control of the SV40 early region promoter or the MSV LTR (7, 8). Following cotransfection with the RSV LTR-*cripto* plasmid and the pSV2*neo* plasmid and subsequent selection in G418-containing medium for 3 weeks, 4 of 22 G418-resistant individual colonies were randomly selected and expanded into cell lines. The presence of the plasmid *cripto* cDNA in the G418-resistant NOG-8 clones was evaluated by Southern blot analysis. High molecular weight DNA was extracted from NOG-8 cells, from NOG-8 *neo* cells, and from three NOG-8 *cripto* clones. Following digestion with *Eco*RI and hybridization with a ^{32}P-labeled human *cripto* cDNA insert, a major 4.8-kilobase DNA restriction fragment and four minor fragments (8, 6.6, 3, and 1.7 kilobases) could be detected in NOG-8 cells and in NOG-8 *neo* cells (Fig. 1, *top*). These fragments are apparently derived from the endogenous mouse *cripto* gene and from some *cripto*-related pseudogenes.[3,4] In constrast, NOG-8 *cripto* clone 5, 8, and 10 cells possessed additional restriction fragments ranging from 1.7 to 9 kilobases long, suggesting that random integration and rearrangements of the plasmid have occurred in these clones. In addition, the greater intensity of some of these restriction fragments in NOG-8 *cripto* clone 5, 8, and 10 cells suggests that multiple copies of the plasmid DNA have been integrated into these cells. To ascertain whether the integrated plasmid *cripto* cDNA was correctly transcribed, poly(A)$^+$ RNA was isolated from these three NOG-8 *cripto* clones, from NOG-8 cells, and from NOG-8 *neo* cells and analyzed by Northern blotting after hybridization with a ^{32}P-labeled human *cripto* cDNA insert. A specific 4.5-kilobase *cripto* mRNA that is consistent with the expected size for the expres-

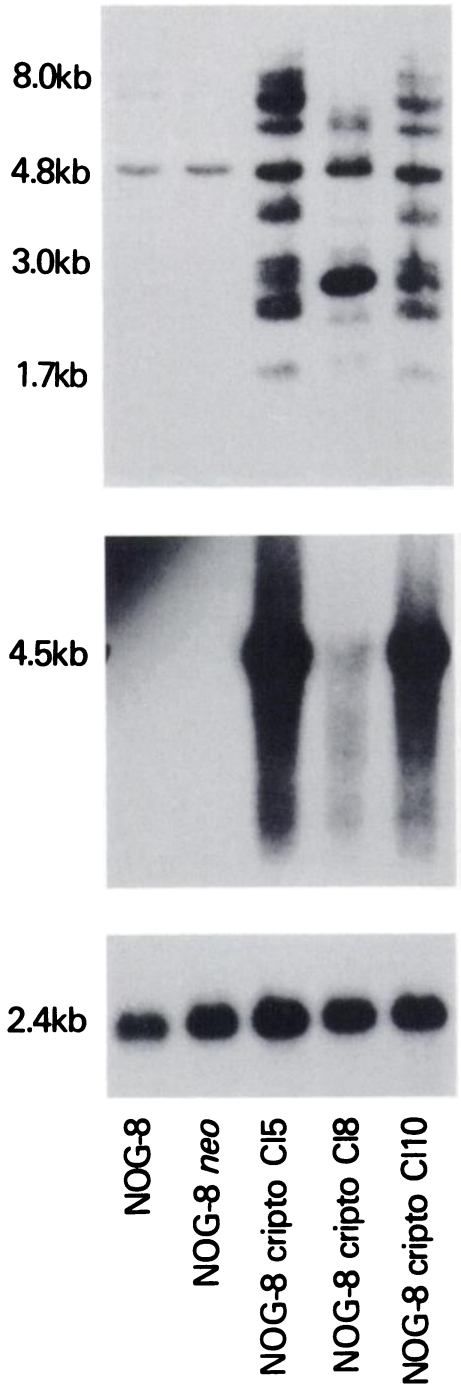

Fig. 1. Southern blot analysis of DNA and Northern blot analysis of poly(A)$^+$ RNA extracted from NOG-8, NOG-8 *neo*, and NOG-8 *cripto* clone 5, 8, and 10 cells. *Top*, high molecular weight DNA (10 μg) was digested with *Eco*RI before being fractionated on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled human *cripto* cDNA insert. *Middle* and *bottom*, poly(A)$^+$ RNA (10 μg/lane) was fractionated on a 1.2% denaturing agarose gel, transferred to nylon membrane, and hybridized to a ^{32}P-labeled human *cripto* cDNA insert (*middle*) or a human β-actin cDNA insert (*bottom*). *kb*, kilobases.

[4] R. Dono and M. G. Persico, unpublished results.

sion vector plasmid *cripto* mRNA could be observed in NOG-8 *cripto* clone 5, 8, and 10 cells (Fig. 1, *middle*). The highest levels of *cripto* mRNA expression were detected in NOG-8 *cripto* clone 5 and 10 cells. No endogenous 2.2-kilobase *cripto* mRNA could be found in NOG-8 or NOG-8 *neo* cells. Equivalent amounts of RNA were loaded on the gel since the levels of a 2.4-kilobase β-actin mRNA were comparable in all of the cell lines (Fig. 1, *bottom*).

One characteristic feature of transformed cells *in vitro* is their ability to grow as colonies in semisolid medium. Therefore, to determine if expression of *cripto* in the NOG-8 *cripto* transfectants is functionally significant for the acquisition of a transformed phenotype, the ability of these cells to grow in anchorage-independent conditions was tested. As shown in Table 1 and Fig. 2, NOG-8 and NOG-8 *neo* cells were unable to clone in soft agar, whereas the four NOG-8 *cripto* clones were able to form colonies under these growth conditions. Furthermore, two NOG-8 *cripto* transfectants, NOG-8 *cripto* clone 5 and clone 10 cells that express the highest levels of *cripto* mRNA, are able to grow as very large colonies in soft agar (Table 1). In addition, they exhibit a cloning efficiency that is approximately comparable to that of NOG-8 cells which have been transformed by an activated human c-Ha-*ras* protooncogene, NOG-8 *ras* cells (11). To determine whether *cripto* could modify the anchorage-dependent growth of NOG-8 cells that are expressing high levels of this gene, NOG-8 cells and NOG-8 *cripto* clone 5 cells were grown in serum-free medium in the absence of exogenous EGF. Fig. 3 shows that NOG-8 *cripto* clone 5 cells exhibit a 2.5- to 3-fold increase in their growth rate after 4 days in PC-1 serum-free medium devoid of EGF as compared to NOG-8 cells.

The ability of cells to clone in soft agar is one index of *in vitro* transformation but does not always correlate with tumorigenicity *in vivo* (15). To determine whether NOG-8 *cripto* clones that are able to grow as colonies in semisolid medium are also tumorigenic, 5×10^6 cells from NOG-8, NOG-8 *neo*, NOG-8 *ras*, and NOG-8 *cripto* clone 5, 8, and 10 cells were injected s.c. into the dorsal flank of 10 nude mice for each cell line. NOG-8 *ras* cells formed locally invasive, undifferentiated mammary carcinomas that were palpable within 2 to 3 weeks after injection in 10 of 10 animals (data not shown). In contrast, no tumors could be detected during a 4-month observation period in any of the mice that had been given injections of NOG-8 cells, NOG-8 *neo* cells, or the NOG-8 *cripto* transfectants.

In summary, this is the first study to demonstrate that expression of a human *cripto* cDNA in an immortalized population of mammary epithelial cells is capable of transforming these cells *in vitro*. *cripto* may therefore function as a transforming gene in other epithelial cells or in fibroblasts. In this respect, overexpression of *cripto* in NIH-3T3 cells is able to induce focus formation of these cells *in vitro* (1). Nevertheless, expression of *cripto* is not entirely sufficient to lead to a tumorigenic phenotype *in vivo*. These results suggest that additional genetic changes may be necessary to complete the transformation process *in vivo*. For example, transformation of primary rodent embryonic fibroblasts requires the cooperative interaction between an activated c-*ras* gene and a second nuclear oncogene such as c-*myc* (16). This is probably due in large part to the ability of these nuclear oncogenes such as *myc* and E1A to immortalize cells and thereby to sensitize them to the biological effects of *ras*. Alternatively, deletions or loss of expression of a tumor suppressor gene may be necessary in the NOG-8 *cripto* cells to facilitate the formation of tumors *in vivo*. Finally, since *cripto* has the potential of encoding an EGF-related peptide, it is equally plausible that the *cripto* protein may interact with a cell surface receptor that is distinct from the EGF receptor. Two such EGF receptor-related genes, c-*erbB*-2 and c-*erbB*-3,

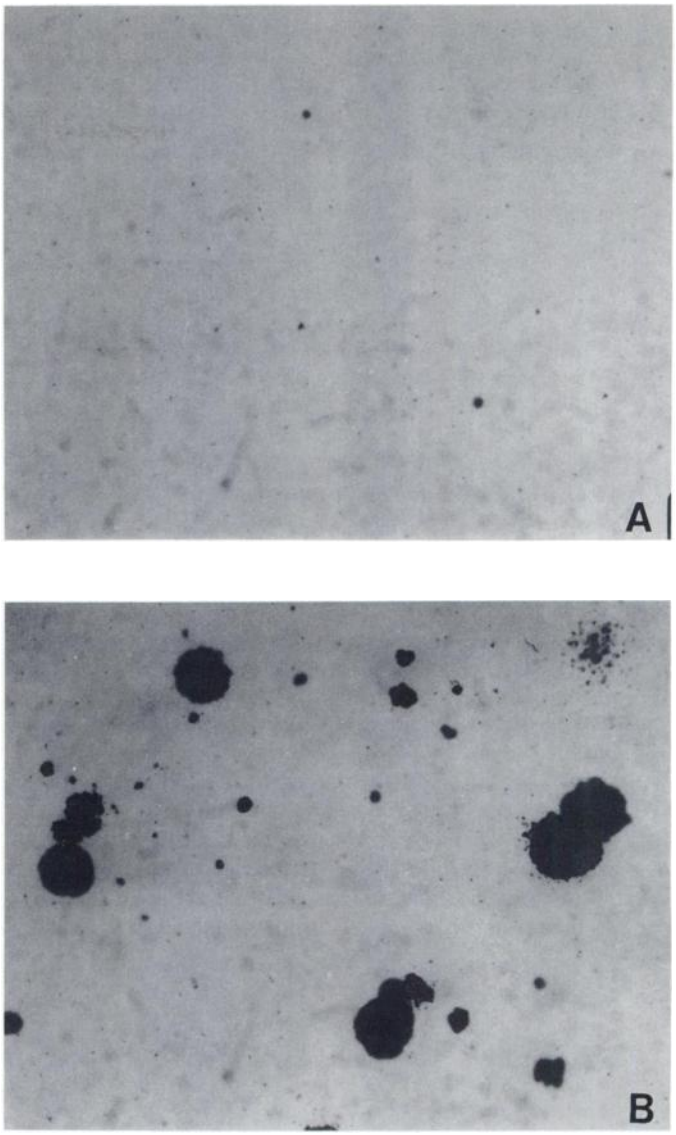

Fig. 2. Morphology of NOG-8 *cripto* colonies in soft agar. *A*, NOG-8 cells. *B*, NOG-8 *cripto* clone 5 cells. Two × 10^4 cells/35-mm dish were plated in soft agar as described in "Materials and Methods."

Table 1 *Anchorage-independent growth in soft agar*

Two × 10^4 cells/35-mm dish were seeded in soft agar as described in "Materials and Methods." After 3 weeks colonies were stained with nitroblue tetrazolium and counted with an Artek 880 colony counter. Results represent the average ± SD of two separate experiments each performed in quadruplicate. NOG-8 *ras* cells were grown in the presence of 1 μM dexamethasone to induce the expression of the point mutated c-Ha-*ras* protooncogene placed under the transcriptional control of the mouse mammary tumor virus LTR, as described previously (11).

Clones	Colonies/dish larger than			
	0.05 mm	0.2 mm	0.5 mm	1 mm
NOG-8	3	0	0	0
NOG-8 *neo*	2	0	0	0
NOG-8 *cripto* Cl 5	1690 ± 80	950 ± 50	306 ± 8	36 ± 1
NOG-8 *cripto* Cl 6	175 ± 25	59 ± 15	4 ± 1	0
NOG-8 *cripto* Cl 8	166 ± 10	117 ± 20	30 ± 4	0
NOG-8 *cripto* Cl 10	925 ± 70	630 ± 80	250 ± 9	38 ± 5
NOG-8 *ras*	2955 ± 75	1950 ± 50	231 ± 9	26 ± 1

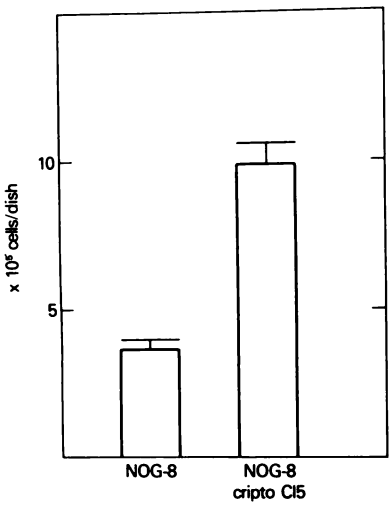

Fig. 3. Anchorage-dependent growth of NOG-8 and NOG-8 *cripto* clone 5 cells (*Cl*) under serum-free medium conditions. Two × 10^4 cells/dish were seeded in 12-multiwell cluster dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After 24 h, the cells were switched to PC-1 serum-free medium for 4 days and then counted with a model ZBI Coulter Counter. Results represent the average ±SD (*bars*) of three different experiments each performed in quadruplicate.

have recently been identified in mammary epithelial cells (17–19). The ligands for these two receptors have not been identified. Although NOG-8 cells express a sufficient complement of EGF receptors such that overexpression of TGF α can lead to their transformation *in vitro* and *in vivo*, these cells may be expressing relatively low levels of a putative receptor for *cripto* thereby impeding complete transformation when a *cripto* expression vector is introduced into these cells (6–8).

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