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GRANT NO: DAMD17-94-J-4286

TITLE: Epidermal Growth Factor-Like Ligands in Breast Cancer

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CONTRACTING ORGANIZATION: La Jolla Cancer Research Foundation

REPORT DATE: September 22, 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 22, 1995	Annual 22 Aug 94 - 21 Aug 95

4. TITLE AND SUBTITLE
Epidermal Growth Factor-Like Ligands in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4286

6. AUTHOR(S)
Eileen D. Adamson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
La Jolla Cancer Research Foundation
La Jolla, California 92037

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The expression of amphiregulin (Ar) and Cripto-1 (Cr-1) genes was assayed in developing mouse mammary glands. Expression was also detected in the developing mouse embryo and embryonic stem (ES) cells and embryonal carcinoma (EC) cells. Cr-1 is present in proliferating differentiated cells and is down regulated in non-proliferating differentiated cells. Ar expression is more ubiquitous than was reported earlier.

The assays were performed using reverse-transcription polymerase chain reaction (RT-PCR), immunoblotting and immunocytochemistry. Difficulties in interpretation were encountered that demanded the preparation of better antibodies. Anti-Ar was made and anti-Cr-1 is in progress.

We will prepare retroviral vectors to over-express Cr-1 and Ar in mammary gland cells and determine their effect in reconstituted glands. For the reduction in Cr-1 expression we will next use gene disruption in ES cells to prepare Cr-1 null mice. This will be a more efficient method to understanding the role of Cr-1 in development and in mammary glands.

14. SUBJECT TERMS
Amphiregulin, Cripto-1, growth factors, expression levels, developmental roles, mammary glands.

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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E. J. Adamson 8/24/95

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(5) INTRODUCTION

The Epidermal Growth Factor (EGF)-like growth factors, Amphiregulin (Ar) and Cripto-1 (Cr-1), are expressed in 77% and 79%, respectively, of human breast carcinomas but are only present in low levels (Ar) or not at all (Cr-1) in the surrounding normal tissue. This project is to determine if these growth factors play a role in tumor formation in the mammary gland where they could play autocrine or paracrine roles.

The experimental approach is a mouse mammary cell transplantation of primary mammary cell cultures that have been infected with retroviruses expressing a variety of forms of the two genes. The cells are injected into the cleared mammary fat pads of recipient syngeneic mice to determine the effect on subsequent development and on the production of tumors. The method has been previously used for other growth factors and other genes, but little is known about the involvement of Ar and Cr-1.

(6) BODY

i) Project to determine the expression of Ar and Cr-1, in postnatal developing virgin, pregnant and lactating mammary glands. In order to provide a normal base-line of the expression of these genes in the mammary gland, we undertook a developmental study using a variety of techniques to detect mRNA and protein. We used RT-PCR to show that the Ar and Cr-1 genes are expressed. We next used quantification of immunoblots (Western) analysis with antibodies (anti-human) obtained from other workers to show that both genes are developmentally and hormonally regulated. For Ar, expression of the 26/30 kDa protein increases gradually from week 3 to 12 of postnatal development, but a large increase occurs upon pregnancy and this is sustained through lactation. For Cr-1 we reported the expression of immunoreactive proteins at 90, 77, 56 and 21 kDa using the anti-human Cr-1 antibodies. Only pregnant and lactating mammary tissue produced the 2i kDa species which is the recognized form of Ar. We believe therefore that the authentic protein is not produced in detectable amounts in virgin mammary glands but is produced and secreted in pregnant and somewhat in lactating glands. These results have been published in Mol. Dev. Biol.(see Appendix).

Immunocytochemistry using the same antibodies confirmed the results of the biochemical analyses. These results made us aware that the antibodies that we are using may cross react with other proteins in mammary cells. It therefore became important to make better antibodies with more reliable specificities if possible.

ii) Project to make antibodies to mouse Ar. We chose a hydrophilic peptide in the Ar sequence, made a synthetic peptide and linked it to KLH to use as an antigen in two rabbits. Both rabbits gave excellent antibodies that react in immunoprecipitation, immunoblotting and immunocytochemistry. The protein detected is about 28 kDa and is found in skin, tongue and mammary glands. It is produced in mammary tumor cell lines as well as F9 Embryonal carcinoma (EC) cell lines. We will now be able to quantify the Ar produced in mammary tumors and normal tissues to determine when and where it is regulated. One observation that needs to be confirmed is that normal human mammary follicle cells stain for AR in a pericellular pattern, while human breast tumor tissues stain in a nuclear fashion. This difference in staining

pattern could indicate a different mode of action in transformed cells (see Appendix Fig 5).

We have also affinity purified the antibody and used it to show strong expression in the mouse 8-cell embryo and blastocyst. We have shown that Ar is expressed by RT-PCR and by immunostaining where the nucleus is the most reactive, in agreement with the literature. In addition, we have shown that antisense oligonucleotides prevent the production of Ar. The biological effect of reduced Ar production is the inhibition of cavitation (a measure of the differentiation of the trophoblast cells). We therefore concluded that Ar plays a role in preimplantation development in vitro. This work is being done in collaboration with Dr. Lynn Wiley (UC, Davis) and her graduate student E. Tsark. This is an important finding since it suggests that there is a distinct developmental role for Ar and this is of interest in understanding the ways in which the expression can become aberrant in tumors.

iii) Project to make antibodies to Cr-1. We were particularly concerned about the results of Cr-1 expression that gave anomalous molecular sizes. We have obtained 3 different antipeptide antibodies from Dr. Graziella Persico, and all give a great many high molecular weight bands on immunoblots of pregnant mammary glands and F9 EC cells which express very high levels of Cr-1. These bands were in addition to the authentic 24 kDa protein band. We are now making a fusion protein in bacteria and have successfully prepared a bacterial clone that makes sufficient Cr-1 protein. This will be extracted, purified on a column and antibodies made in rabbits. It will take another 4 months before new antibody is available. Reliable and specific antibody is necessary to quantitate the level of Cr-1 protein in cells and tissues where the levels are being manipulated.

iv) Project to determine the expression of Cr-1 in stem cells and in mammary cell lines. The distribution pattern of Cr-1 is not known, although it is thought to correlate with the transformed cell. This was confirmed in 1) F9 EC and D3 ES cells which were positive for Cr-1 **(Fig 1)** while their differentiated derivatives (visceral and parietal endoderm, VE and PE) were negative. 2) In mammary tissues, there was very little Cr-1 in virgin glands, extremely high in pregnant and less in lactating tissues **(Fig 2)**. 3) In pregnant mice, there is detectable Cr-1 in placenta, but we do not know if it was derived from secreted forms from the mammary gland. After involution of the mammary gland, Cr-1 expression decreases **(Fig 3)**. There is also a detectable level of Cr-1 in breast tumor tissue from transgenic mice that express the middle T antigen of polyoma virus (Fig 3, mid T). 4) Results that were the reverse of the above were obtained in the CID9 mouse cell line that was derived from a mid-pregnant mouse. These cells differentiate when cultured on Matrigel (basement membrane)while the cells plated on plastic are considered to be less differentiated. Cells on plastic do not express Cr-1, while the differentiated cells on Matrigel secrete milk proteins and express Cr-1 **(Fig 4)**. This is unexpected and suggest that there are two signal pathways for Cr-1 expression, one is via hormonal (progesterone) signals and the other is matrix stimulated. This will need further work to elucidate.

v) Project to make retroviruses to overexpress Cr-1 in mammary cells. We have had little success so far in getting high titer retrovirus in culture medium of PA319 packaging cells. The vector is selectable in hygromycin and uses the metallothionein promoter to drive the expression of Cr-1. We have

consulted Dr. M.L.Cutler who made the original vector and have some useful suggestions to improve activity. These studies are in progress.

vi) Project to reduce the level of Cr-1 expression. We now have an alternate method planned instead of the antisense experiments to reduce the level of expression of the Cr-1 gene in mice. We have been asked to make the targeted inactivation of the Cr-1 gene in mice using a targeting vector made by Dr. Graziella Persico of Naples, Italy. We consider that this is a better way to make mice that do not express Cr-1 and to use these mice as donors or recipients in the mammary transplantation experiments that were described in the original application. This experiment will be started during the next year. It will involve the homologous replacement of an inactive Cr-1 gene in ES cells, the injection of these Cr-1(+/-) cells into mouse blastocysts and hence the preparation of chimeric animals. These will be bred to give heterozygous and then homozygous animals. We suspect that there will be a deleterious effect during development and this could involve the stages and tissues that are known to express Cr-1 , namely, the blastocyst, the primitive streak, the heart and mammary gland. We estimate that it will take at least a year before we will know the best studies to undertake on these animals.

(7) CONCLUSIONS

1. Amphiregulin

a) We have shown that this gene is expressed in mammary gland at all stages of development, and especially during pregnancy and lactation. It is seen in both mammary ducts, lobules and stromal cells of the fat pad.

b) We made an antibody to show that Ar is expressed in adult skin, and all organs that have ectodermal/epidermal organs such as tongue as well as mammary gland. We also detected Ar expression in ovary and testes and embryonic stem cells. It is expressed as early as the 8-cell embryo during preimplantation development. It is therefore more ubiquitous than thought earlier. Its expression does not seem to be useful as a unique marker of the mammary tumor cell, but may be over-expressed in transformed cells. The expression of Ar will be further tested using the new antibodies.

2. Cripto-1

a) This gene is expressed in a much more restricted manner compared to Ar. It seems to be a marker of the proliferating differentiated mammary gland and hence some forms of mammary tumor. It is also expressed in preimplantation blastocysts (not our work) and in ES cells (our findings).

b) Because we were given the opportunity to specifically target the Cr-1 gene in a collaborative experiment, we will next make a Cr-1 null mouse by homologous recombination. If the resulting animals are viable, this will allow us to prepare mammary cells that lack Cr-1 to use in transplantation experiments. This will displace the studies to reduce Cr-1 expression in mammary tissue using antisense retroviruses. This is a much more efficient way of achieving our requirements as well as allowing us to determine the role of Cr-1 in development.

(8) REFERENCES

Kenney, N.J., Huang, R-P., Wu, J-X., Okamura, D., Matheny, W., Kordon, E., Gullick, W.J., Smith, G.H., Salomon, D.S. and Adamson, E.D. (1995). Expression of amphiregulin (Ar) and cripto-1 (Cr-1) in the developing mouse mammary gland. Mol. Reprod. Devel. 41, 277-286.

(9) APPENDIX

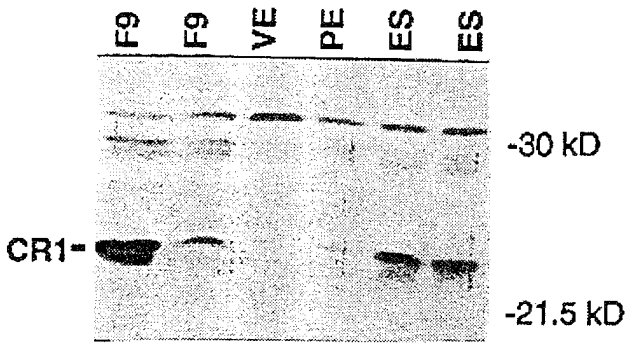


Figure 1. Western blot analysis showing cripto expression in undifferentiated F9 cells (F9) and ES cells (ES) but not in F9 cells differentiated into visceral endoderm (VE) or parietal endoderm (PE).

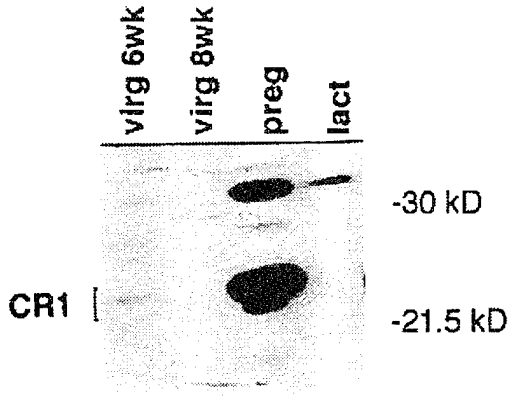


Figure 2. Western blot analysis of the native cripto protein in different developmental stages of the mammary gland and F9 embryonal carcinoma cells.

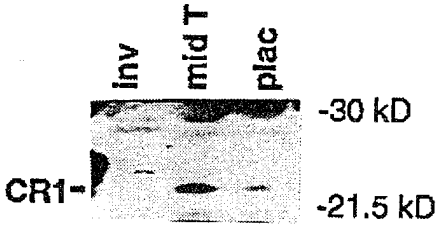


Figure 3. Western blot analysis of the native cripto protein in mammary gland tissue showing cripto is expressed in polyoma middle T antigen breast tumor tissue (mid T) but not in normal breast tissue from retired breeders or mice that have undergone involution (inv). Also shown is weak expression of cripto in the mouse placenta, where it could be located after secretion and transport from its source.

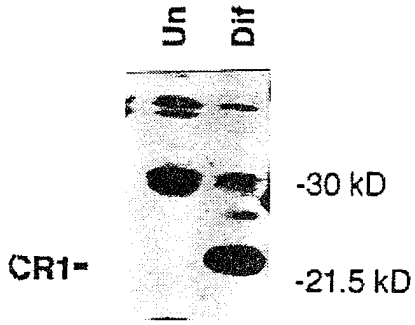
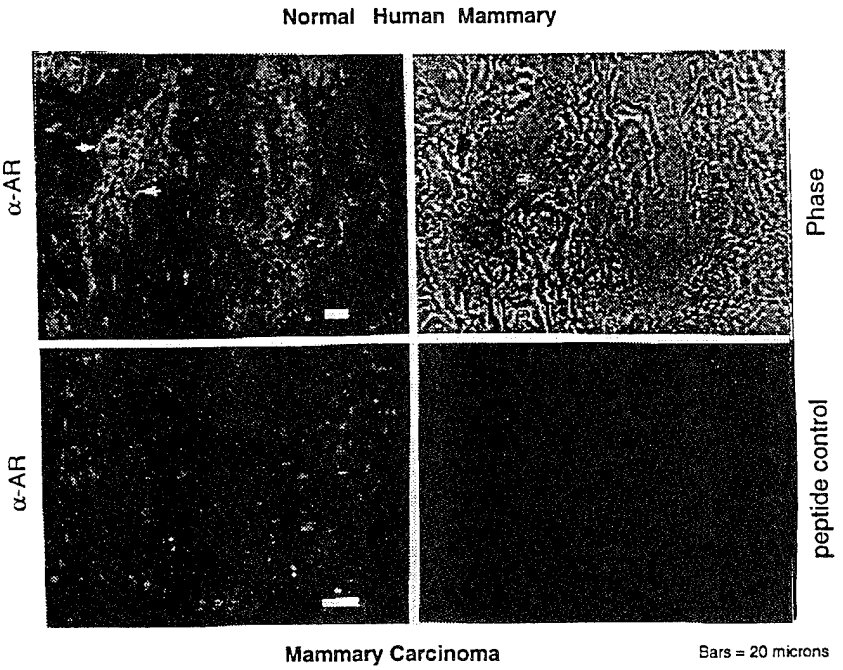


Figure 4. Western blot analysis showing cripto is expressed in the CID-9 cells when differentiated (Dif) but not when undifferentiated (Un).

(9) APPENDIX

Figure 5. Detection of AR in human mammary gland by immunofluorescence. Frozen sections of normal human breast tissue (upper panels) were detected with our rabbit anti-mouse Ar affinity purified antibody. Breast cancer samples were also positive for AR but stained largely in nuclei (lower left). An irrelevant antibody did not stain either of these tissues (lower right panel).



MOLECULAR REPRODUCTION AND DEVELOPMENT 41:277–286 (1995)

Detection and Location of Amphiregulin and Cripto-1 Expression in the Developing Postnatal Mouse Mammary Gland

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ABSTRACT Amphiregulin (Ar) and Cripto-1 (Cr-1) are growth promoting peptides that share amino acid sequence homology with epidermal growth factor (EGF). The present study examined Ar and Cr-1 mRNA and protein expression during various stages of C57BL/6 mouse mammary morphogenesis. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect transcripts for Ar and Cr-1 at all stages of mammary development. Immunocytochemical (ICC) localization demonstrated that in virgin 4-week to mature 12-week-old mouse fourth inguinal mammary gland, Ar and Cr-1 are expressed in the stromal cells, luminal epithelial cells, and myoepithelial cells of the branching ducts. Ar, and to lesser extent Cr-1, were also found in the epithelial cap cells and in the luminal epithelial cells of the advancing terminal end bud (TEB) from virgin 4-week and 6-week-old mice. Western blot analysis demonstrated that both Ar (28 and 26 kDa) and Cr-1 (90, 67, 56, and 21 kDa) proteins are expressed in virgin, 13.5 day midpregnant and in the 14 day lactating mammary gland. In addition, Ar and Cr-1 are associated with developing alveolar structures as determined by ICC. These results imply that together with EGF and transforming growth factor alpha (TGFα), Ar and Cr-1 may play salient roles as modifiers in the morphogenesis and differentiation of the mammary gland.

Key Words: EGF-like ligands, Postnatal development, RT-PCR, Immunocytochemistry, Immunoblotting

INTRODUCTION

Morphogenesis and differentiation of the rodent mammary gland are highly complex series of events that occur largely postnatally (Daniel and Silberstein, 1987). The first stages of development of the mammary gland begin at day 10 to day 11 after fertilization. On day 17 of gestation, a primitive outline of the gland is observed (Daams et al., (1987). From this point until approximately 4 weeks of age, the mammary gland is essentially quiescent in growth. At 4 weeks of age, when ovarian hormone levels increase, the invasive epithelial structures referred to as terminal end buds appear and proliferate from the nipple region, forming a network of epithelial ducts that fill the fat pad. During this process, the rapid and extensive proliferation of ductal epithelial structures either ceases upon encountering other ductal structures or when the end buds reach the periphery of the fat pad. A second round of growth and differentiation occurs during pregnancy. This phenomenon initiates development of lobular-alveolar structures that are capable of synthesizing milk proteins.

Growth factors play an important role in regulating the growth and development of the mammalian mammary gland (Dembinski and Shiu, 1987). Transforming growth factors such as TGFβ1, TGFβ2, TGFβ3, and TGFα, as well as EGF, are potent inhibitors and mitogens for mammary epighelial cells, and have been shown to be differentially expressed during the development of the mouse mammary gland (Silberstein and Daniel, 1987; Robinson et al., 1991; Daniel et al., 1987; Coleman et al., 1988; Snedeker et al., 1991; Vonderhaar, 1987; Brown et al., 1989; Liscia et al., 1990). Moreover, the expression of some of these peptides is regulated by ovarian hormones and probably serves as an important autocrine and paracrine modulator that can regulate end bud, ductal, and alveolar growth and development.

Human amphiregulin (AR) was initially isolated from the human breast cancer cell line MCF-7 after treatment with the phorbol ester, TPA (Shoyab et al., 1988). Fully processed secreted AR is 78–84 amino acids in length and has a six cysteine motif similarly located in EGF and TGFα (Shoyab et al., 1989). AR binds to the epidermal growth factor receptor (EGFR)

Abbreviations: Ar, amphiregulin; RT-PCR, reverse transcription polymerase chain reaction; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; TGFα, transforming growth factor alpha; ICC, immunocytochemistry; Cr-1, Cripto; TPA, 12-o-tetradecanoylphorbol-13-acetate

Received August 22, 1994; accepted October 20, 1994.

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(Shoyab et al., 1989) and also induces tyrosine phosphorylation of the EGFR and $p185^{erbB2}$ through an EGFR-dependent transphosphorylation (Johnson et al., 1993).

AR is expressed in several tissues, including human ovary, testis, placenta, cardiac muscle, lung, spleen, kidney, pancreas, breast, stomach, colon, and porcine corpora lutea (Plowman et al., 1990; Johnson et al., 1992; Kennedy et al., 1993). AR is a potent mitogen for normal human mammary epithelial cells and for several human mammary epithelial cell lines (Shoyab et al., 1988; Li et al., 1992; Kenney et al., 1993; Normanno et al., 1994). In addition, reduction of AR levels by specific antisense oligonucleotides or by addition of exogenous heparin to human mammary epithelial cells leads to a reduction in cell growth in vitro (Li et al., 1993; Kenney et al., 1993; Normanno et al., 1994). Several estrogen-responsive and estrogen nonresponsive human breast cancer cell lines and approximately 80% of human primary breast carcinomas overexpress AR (LeJeune et al., 1993; Normanno et al., 1994; Qi et al., 1994). AR is an estrogen-inducible protein, and its expression is also enhanced following transformation of mammary epithelial cells by point-mutated cHa-ras oncogene or by overexpression of c-erb-B2 (Normanno et al., 1993, 1994).

Human Cripto-1 (CR-1) was initially isolated and cloned from the undifferentiated human embryonal carcinoma cell line NTERA 2 clone D1 (NT2D1) (Ciccodicola et al., 1989). The mouse Cr-1 gene was cloned from F9 mouse embryonal carcinoma cells and encodes a 171 amino acid protein that exhibits 93% amino acid homology to human CR-1 and, like AR, EGF, and TGFα, has a conserved EGF-like cysteine residue repeat (Dono et al., 1993). However, the cysteine region that comprises the A-loop has been truncated; therefore, it is unlikely that Cr-1 binds to the EGFR. The cognate receptor for Cr-1 has not been identified. Recently Brandt et al. (1994) have demonstrated that the CR protein can function as a mitogen for normal and transformed human mammary epithelial cells through an EGFR-independent pathway.

Cr-1 gene expression occurs in a restricted number of tissues during mouse development in a stage-specific manner and is initiated prior to gastrulation (Dono et al., 1993). Cr-1 mRNA expression can be detected in adult mouse spleen, heart, lung, and brain, but not in testis, ovary, intestines, liver, stomach, muscle, kidney, or seminal vesicles (Ciccodicola et al., 1989; Dono et al., 1993). CR may be involved in tumorigenesis since overexpression of human CR-1 in NIH 3T3 cells or in NOG-8 nontransformed mouse mammary epithelial cells leads to their ability to grow in soft agar (Ciccodicola et al., 1989; Ciardiello et al., 1991). Overexpression of CR-1 has been detected by ICC in 80% of breast carcinomas and is infrequently expressed in adjacent noninvolved breast epithelium (Normanno et al., 1993; Qi et al., 1994). Also, several estrogen responsive and nonresponsive human breast cancer cell lines express CR-1 mRNA and protein (Normanno et al., 1993). In the present study, we have examined the expression and localization of Ar and Cr-1 in the developing mouse mammary gland.

MATERIALS AND METHODS

Cell Lines and Primary Cultures

The HC-11 mouse mammary epithelial cell line was obtained from Dr. Nancy Hynes (Freidrich-Meischer Institute, Basel, Switzerland) and was grown in Dulbecco's modified Eagle's medium (DMEM) /F-12 (1:1) supplemented with 10% fetal bovine serum (FBS) and EGF (10 ng/ml) at 37°C in 5% CO_2. Mouse F9 teratocarcinoma embryonal carcinoma cells were obtained from Dr. M.G. Persico (International Institute of Genetics and Biophysics, CNR, Naples, Italy) and were maintained on gelatin-coated plastic dishes in DMEM supplemented with 10% FBS at 37°C in 5% CO_2. Primary mouse mammary epithelial cell cultures were obtained by collagenase digestion of mammary glands excised from 12-week-old mice and incubated 24 hr at 37°C. Mammary cells were then plated onto gelatin-coated 100 mm plastic dishes and maintained in Dulbecco's minimal essential medium supplemented with 10% FBS, penicillin, streptomycin, EGF (10 ng/ml), and insulin (4μg/ml). Mammary epithelial cells were sequentially trypsinized and plated to eliminate fibroblasts present in cultures.

Animals

Pathogen-free virgin, pregnant, and lactating female C57BL/6 mice were bred in the foundation's facility. All mice were given food and water ad libitum and housed under a 12-hr light, 12-hr dark cycle. Before each experiment mice were euthanized with CO_2 and mammary tissue was dissected out for multiple analyses. Tissues were frozen in liquid N_2 and stored at -80°C.

Antibodies

The primary antibodies used were rabbit Ab-2 raised against Ar 26-44 peptide and affinity purified with immobilized peptide (Johnson et al., 1993; Saeki et al., 1992) and CR67, a rabbit affinity-purified antibody directed against residues 97–113 of the human CR-1 protein, a peptide that is conserved in the mouse (Saeki et al., 1992).

Immunocytochemistry (ICC)

The #4 inguinal mouse mammary glands were obtained from 3-, 4-, 6-, 8-, or 12-week-old virgin mice or from 13.5 day pregnant or 14 day lactating mice and were embedded in OCT embedding medium (optimal cutting for frozen tissues, Tissue-Tek, Miles Inc., Ekhart, IN), and snap froxen and stored at -80°C. Then 10–15 μm sections from OCT-embedded blocks of tissue were fixed in 100% methanol. Endogenous peroxidase activity was inactivated with 0.03% H_2O_2 in methanol. Nonspecific binding was blocked with 5% normal goat serum, after which the sections were incubated for 12 hr with 1 μg/ml of the anti-Ar antibody or with 1 μg/ml of the anti-Cr-1 antibody at 4°C. Antigen/antibody com-

plexes were detected by subsequent incubation with biotinylated goat anti-rabbit antibody at a 1:1,000 dilution and avidin-biotin complex conjugated to horseradish peroxidase with 3-3'-diaminobenzidine-4-HCl (DAB) as the chromogen for visualization or with biotinylated goat anti-rabbit antibody at a 1:1,000 dilution and alkaline phosphatase for immunofluorescence utilizing Vector Red as the chromogen (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The slides were then lightly counterstained with hematoxylin.

Western Blotting

Western blot analysis was performed on lymph node–free, #4 inguinal mouse mammary glands obtained from 4-, 6-, 8-, 10-, and 12-week-old virgin mice, and from 13.5 day pregnant or 14 day lactating mice. Briefly, glands were homogenized in 1 ml hypotonic buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM PMSF, and 20 μg/ml aprotinin). Extracts (40 μg protein) were mixed with Laemmli sample buffer and then boiled and resolved on 15% SDS/PAGE gels. Equivalent loading of protein samples was determined by staining a parallel gel with Coomassie blue. The gel was then electrophoretically transferred to nitrocellulose, and nonspecific binding was blocked by preincubating the blots in phosphate buffered saline containing 5% (w/v) nonfat dried milk and 0.2% (v/v) Nonidet P-40 (Sigma, St. Louis, MO). Amphiregulin was detected using Ab-2 (1 μg/ml) or was blocked by preabsorbing the antibody against a fivefold excess of the synthetic Ar peptide 26-44 for the control and Cripto-1 was detected using CR67 (1 μg/ml) or preabsorbed against the peptide 97–113. The blots were then incubated with horseradish peroxidase-conjugated goat antirabbit immunoglobin at a 1:1,000 dilution (Sigma) and the proteins were visualized with ECL Western blotting detection reagents (Amersham, Arlington Heights, IL).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from stored frozen tissues by the guanidine isothiocyanate RNAzol B method (Cinna/Biotecx Laboratories, Houston, TX) (Chomczynski and Sacchi, 1987). Total RNA (2.5 μg) was incubated with 100 pM of oligo-dT12-18 (Pharmacia-LKB, Alameda, CA) at 65°C for 2 min in 10.5 μl of diethyl pyrocarbonate (DEPC)-treated water. After cooling to room temperature, 20 μl of 1× reverse transcription buffer (2 μl 10× RT buffer Biolabs; 5 μM dNTPs; 10 μM DTT; 20 units RNasin; Promega, Madison, WI) and 200 units of Moloney murine leukemia virus reverse transcriptase (N.E. Biolabs, Beverly, MA) were added and incubated at 36°C for 1 hr. PCR amplifications were carried out by using one tenth of the reverse-transcribed RNA. The PCR reaction mixture contained 50 mM KCl, 10 mM Tris, pH 8.4 (25°C), 1.5 mM $MgCl_2$, 10 μM of each primer, 100 μM of each dNTP, and 2.5 units of TaqI polymerase (Perkin Elmer, Norwalk, CT) in a final volume of 50 μl. Primers were 5' CTGTTGCTGCTGGTCTTAGG 3' sense and 5' AGAGTTCACTGCCAGAAGGC 3' antisense to amplify a 172 bp fragment of the mouse Ar gene. This portion of the gene is interrupted by an intron, so that any DNA present in the RNA extract would not give the correct sized DNA (Sonoda et. al., 1992). Furthermore, this fragment has a unique *BanII*/*SacI* site that was confirmed by restriction endonuclease digestion of the amplified products. To detect mouse Cr-1 transcripts, primers were designed to amplify a 334 bp fragment of the mouse Cr-1 gene as previously dexcribed (Dono et al., 1993). As an internal control, β-actin primers were used as previously described (Wiley et al., 1992). The fragments were separated on a 1.2% agarose gel containing ethidium bromide.

RESULTS

Detection of Amphiregulin and Cripto-1 mRNA in the Developing Mouse Mammary Gland

Previous evidence indicates that Ar is expressed in nontransformed human mammary epithelial cells (Li et al., 1992; Kenney et al., 1993; Normanno et al., 1994). To determine if this expression pattern is unique to human mammary epithelial cells in vitro, analysis for the expression of mouse Ar mRNA transcripts in developing mouse mammary gland was performed by RT-PCR. Ar transcripts were detected in virgin 4-, 6-, 8-, and 12-week-old mammary glands in addition to 13.5 day midpregnant and 14 day lactating tissue (Fig. 1A) as a product that corresponded to the predicted 172 bp Ar target sequence. An immortalized nontransformed mouse mammary cell line HC-11 (Danielson et al., 1984) and mouse F9 embryonal carcinoma cells were used as positive controls (Fig. 1A). There was no difference in the level of Ar transcripts at any of the stages that were examined (Fig. 1A) because we utilized conditions that allowed detection but not quantification. As a negative control, total RNA from 12 week, midpregnant, lactating and 12-week-old minus reverse transcriptase did not give a signal. For a positive control, all tissues expressed β-actin (Fig. 1C and 1E).

Detection of mouse Cr-1 has not yet been examined in immortalized mouse mammary epithelial cell lines or in the developing mouse mammary gland. Using RT-PCR, we determined that Cr-1 transcripts are produced during various stages of mammary gland morphogenesis. Similar to Ar, Cr-1 transcripts were detected in virgin 4, 6, 8, and 12 week mammary glands and in 13.5 day midpregnant and lactating mammary glands (Fig. 1B) as a product that corresponded to the predicted 334 bp Cr-1 fragment (Dono et al., 1993). HC-11 mouse mammary cell line (Danielson et al., 1984) and mouse F9 embryonal carcinoma cells were used as positive controls; 4-month-old kidney, liver, intestine, and stomach, and F9 cells minus reverse-transcriptase, were used as negative controls (Fig. 1B and 1D) (Dono et al., 1993). As for Ar, there was no change in the

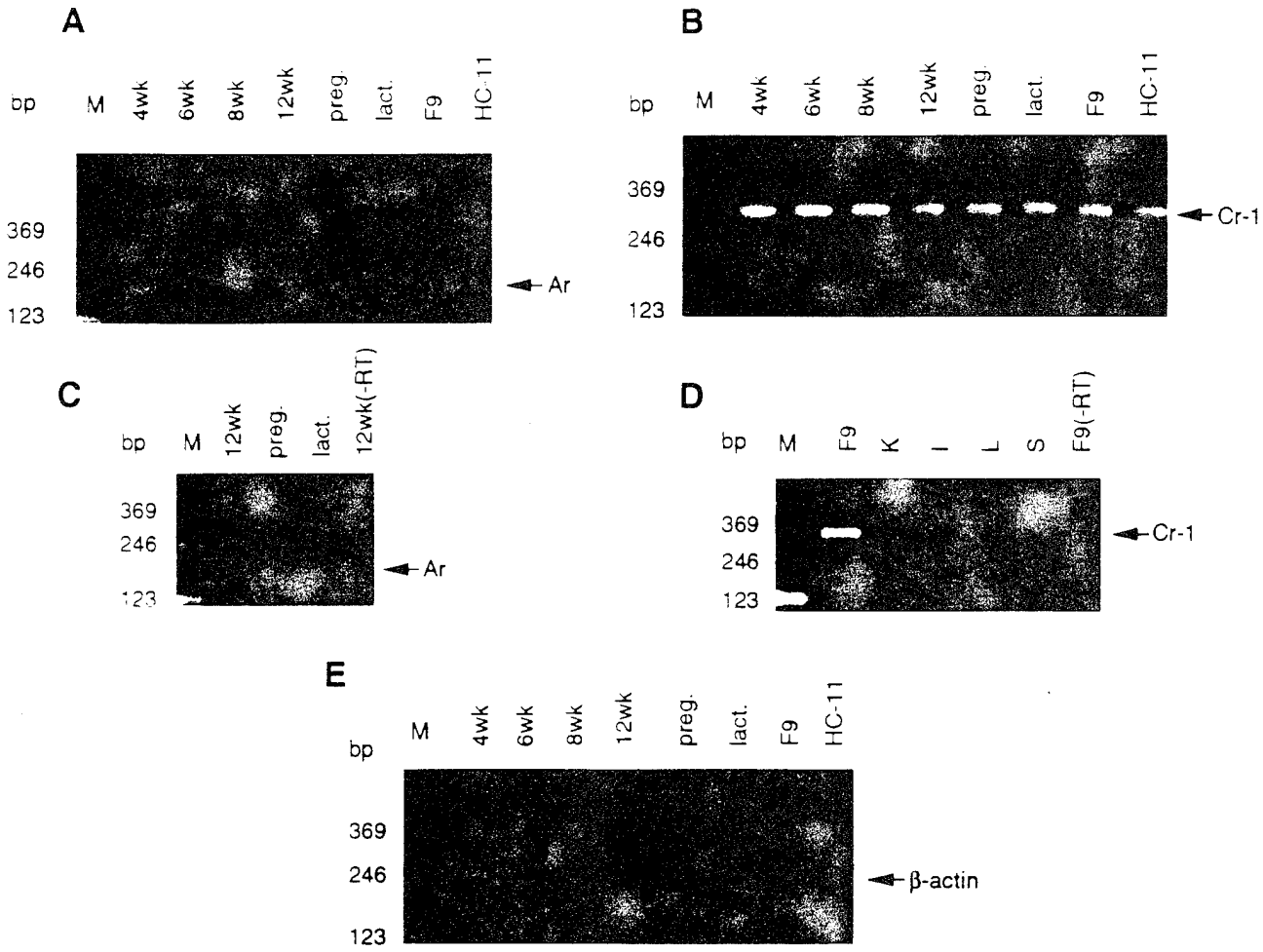


Fig. 1. A. RT-PCR detection of amphiregulin mRNA in normal developing murine mammary gland, murine mammary cell line HC-11, and teratocarcinoma cell line F9. A band of the expected 172-base pair size (arrow) was amplified in each stage of virgin, midpregnant, and lactating glands as well as the HC-11 cell line and teratocarcinoma cell line F9. **B.** RT-PCR detection of cripto-1 mRNA in normal developing murine mammary gland, mouse mammary cell line HC-11, and teratocarcinoma cell line F9. A band of the expected 334-base pair size (arrow) was amplified in each stage of the virgin, midpregnant, and lactating glands, and the HC-11 and F9 cell lines. **C.** Samples of total RNA from 12-week-old mammary glands were subjected to RT-PCR in the absence of RT as a negative control and were analyzed in parallel with RNA from midpregnant, lactating, and 12-week-old glands for expression of Ar. The arrow indicates a 172 bp cDNA product in 12-week-old, midpregnant, and lactating glands but not in samples from 12-week-old glands without reverse transcriptase. **D.** Total RNA from F9 cells with and without RT were subjected to RT-PCR analysis for Cr-1 together with samples from 4-month-old mouse kidney, intestine, liver, and stomach. The arrow indicates the 334 bp transcripts in F9 cells but not in samples from 4-month-old kidney (K), intestine (I), liver (L), stomach (S), and F9 cell extracts without reverse transcriptase. **E.** RT-PCR detection of the expected 245-base pair product (arrow) of the β-actin protein detected in all samples. M, marker; bp, base pair for A–E.

levels of Cr-1 transcripts detected from any stage using these conditions (Fig B).

Expression of Amphiregulin and Cripto-1 Protein During Various Stages of Mouse Mammary Gland Development

To confirm that Ar and Cr-1 mRNA expression correlates with expression of the corresponding proteins, whole tissue extracts were prepared from mammary glands at various stages and were examined by Western blot analysis. Glands were homogenized and the extracts were run on 15% SDS-PAGE gels. After transfer to nitrocellulose, an affinity purified polyclonal antibody against the 26–44 amino acid region of the mature Ar protein was utilized to detect Ar. This antibody does not crossreact with either EGF or TGFα (Johnson et al., 1992), and when preincubated with the Ar peptide immunogen interaction with the antigenic sites was completely blocked (Fig. 2C). As shown in Figure 2A, a 28 kDa protein in the virgin, pregnant, and lactating mammary gland was detected with this antibody. Moreover, in the pregnant and lactating gland, a 26 kDa species was also detected. These species are close to the size of Ar, which was detected in the conditioned medium from TPA-treated MCF-7 human breast cancer cells and from GEO colon carcinoma cells (Shoyab et al., 1989; Johnson et al., 1992). The 28 kDa species, scanned by densitometry, was found to be in-

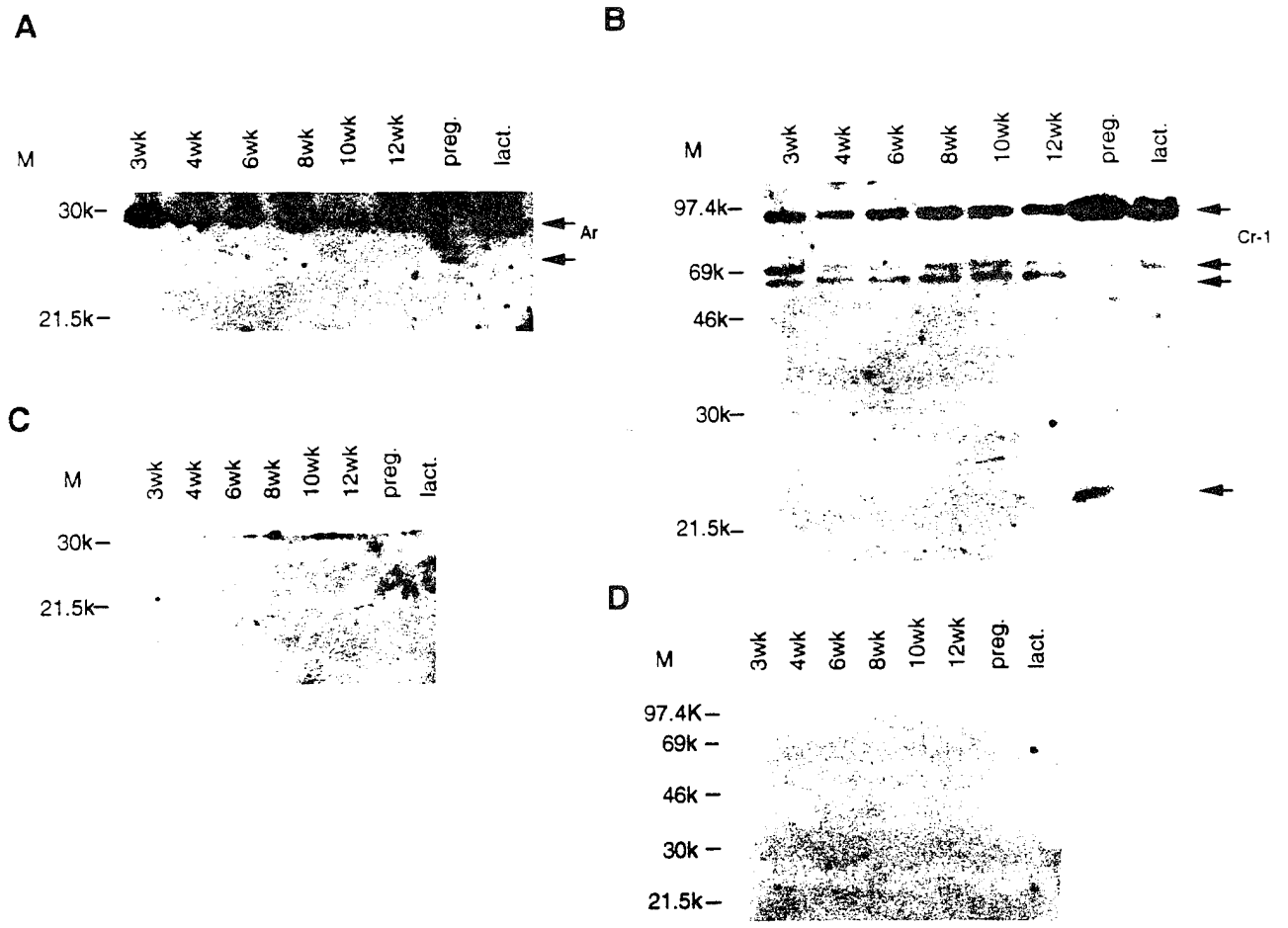


Fig. 2. A. Western blot detection of amphiregulin protein in virgin, pregnant, and lactating stages of the mouse mammary gland. The arrow indicates 28 and 26 kDa species. All lanes were loaded with 40 μg total protein. **B.** Western blot detection of cripto-1 protein in virgin, pregnant, and lactating stages of the mouse mammary gland. The arrow indicates 90, 67, 56, 21 kDa species. All lanes were loaded with 40 μg total protein as described in the methods section. **C.** Western blot control using anti-amphiregulin antibody preabsorbed with the Ar peptide 26-44 immunogen in virgin, midpregnant, and lactating stages of the mouse mammary gland. All lanes were loaded with 40 μg total protein. **D.** Western blot control using anti-cripto-1 antibody preabsorbed with the Cr-1 peptide 97-113 immunogen in virgin, midpregnant, and lactating stages of the mouse mammary gland. All lanes were loaded with 40 μg total protein.

creased approximately 2.5-fold in midpregnant and lactating mammary gland extracts when compared to the level of this species in 3-12 week mammary gland extracts (Fig. 2A).

Mammary gland extracts were also assayed for Cr-1 protein. After transfer to nitrocellulose, a polyclonal affinity purifies antibody against the 97-113 region of mature human Cr-1 protein was reacted with the membrane blot. This antibody does no crossreact with EGF, TGFα, or Ar (Saeki et al., 1992) and when incubated with the Cr-1 17-mer synthetic peptide immunogen, interaction with antigenic sites were completely blocked (Fig. 2D). As shown in Figure 2B, several prominent protein bands were observed with the anti-Cr-1 antibody with MW approximately 94, 67, 56 and 21 kDA. Further, densitometric scanning of bands on lighter exposures of immunoblots indicated that the 94 kDa species increased approximately 2.4-fold from 3 to 6 weeks compared to 8- to 12-week-old mammary gland extracts and more than 20-fold in the midpregnant and lactating mammary glands. In addition, a 21 kDa protein that reacted with the anti-Cr-1 antibody was found in extracts prepared from midpregnant and lactating glands but not in the virgin gland. For both Ar and Cr-1, quite similar levels were detected in glands from 3- to 12-week-old mice. Since there is very little epithelium in 3-week-old glands (before hormonal induction of growth), this indicates that the stroma is a major source for these growth factors at this early stage.

Immunolocalization of Amphiregulin and Cripto-1 in the Developing Mouse Mammary Gland

Localization of Ar or Cr-1 was assessed by immunofluoresence and immunoperoxidase staining of frozen sections obtained from different stages of mammary gland development. At 4 weeks Ar immunoreactivity was detected in various cell types. Control preimmune

IgG or postimmune anti-Ar antibody that was preabsorbed with the 19-mer peptide immunogen failed to exhibit staining (Fig. 3A, curved arrow). More importantly, in virgin 4- and 5-week-old terminal end bud structures from C57BL/6 and FVB/N mice, immunoreactive Ar was detected in the cap cells (Fig. 3B, large solid arrowhead), luminal epithelial cells (Fig. 3C and 4A, medium arrowheads), and myoepithelial cells (Figs. 3D and 4A and large arrows). In the stromal region adjacent to the epithelial structures in 8- and 12-week-old mammary glands (Figs. 3C and 3D), adipocytes (open arowhead), and fibroblasts (thin arrows) were positive for Ar. Secondly, in pregnant and lactating glands, immunoreactive Ar was heavily distributed throughout the mammary gland, which was consistent with our Western blot analysis of Ar expression. In the pregnant mouse mammary gland of C57BL/6 mice, alveolar cells were also positive for Ar (Fig. 4D and F, large arrowhead).

Immunoreactive Cr-1 protein in the virgin mammary epithelium from C57BL/6 and FVB/N mice are illustrated in Figures 3F–H and 4B. At 4 weeks, Cr-1 immunoreactivity was detected in various cell types. Control sections incubated with Cr-1 antibody that had been preabsorbed with the peptide immunogen 97-113 failed to show reactivity, as shown in Figure 3E (curved arrow). In the virgin 4-, 5-, and 8-week-old mammary gland from C57BL/6 and FVB/N mice (Figs. 3F–H and 4B), Cr-1 immunoreactivity was detected in the luminal epithelial cells (Fig. 3H, thin arrow; Fig. 4B, arrowhead) and myoepithelial cells (Figs. 3G and 4B, large arrows) and was occasionally detected in the cap cell population (Fig. 3F, large arrowhead) in 4-week-old mammary gland terminal end bud structures. Cr-1 reactivity was also frequently detected in the lumen of 12-week-old mammary epithelial tubules. Like Ar, Cr-1 was also detected in midpregnant and lactating glands with dense perinuclear localization in the alveolar cells (Figs. 4H and J, large arrowhead). In accordance with previous findings of Dono et al. (1993), cells that compose the vascular tissue were negative for immunoreactive Cr-1 (data not shown).

Localization of Amphiregulin and Cripto in Primary Cultures of Mouse Epithelial Cells

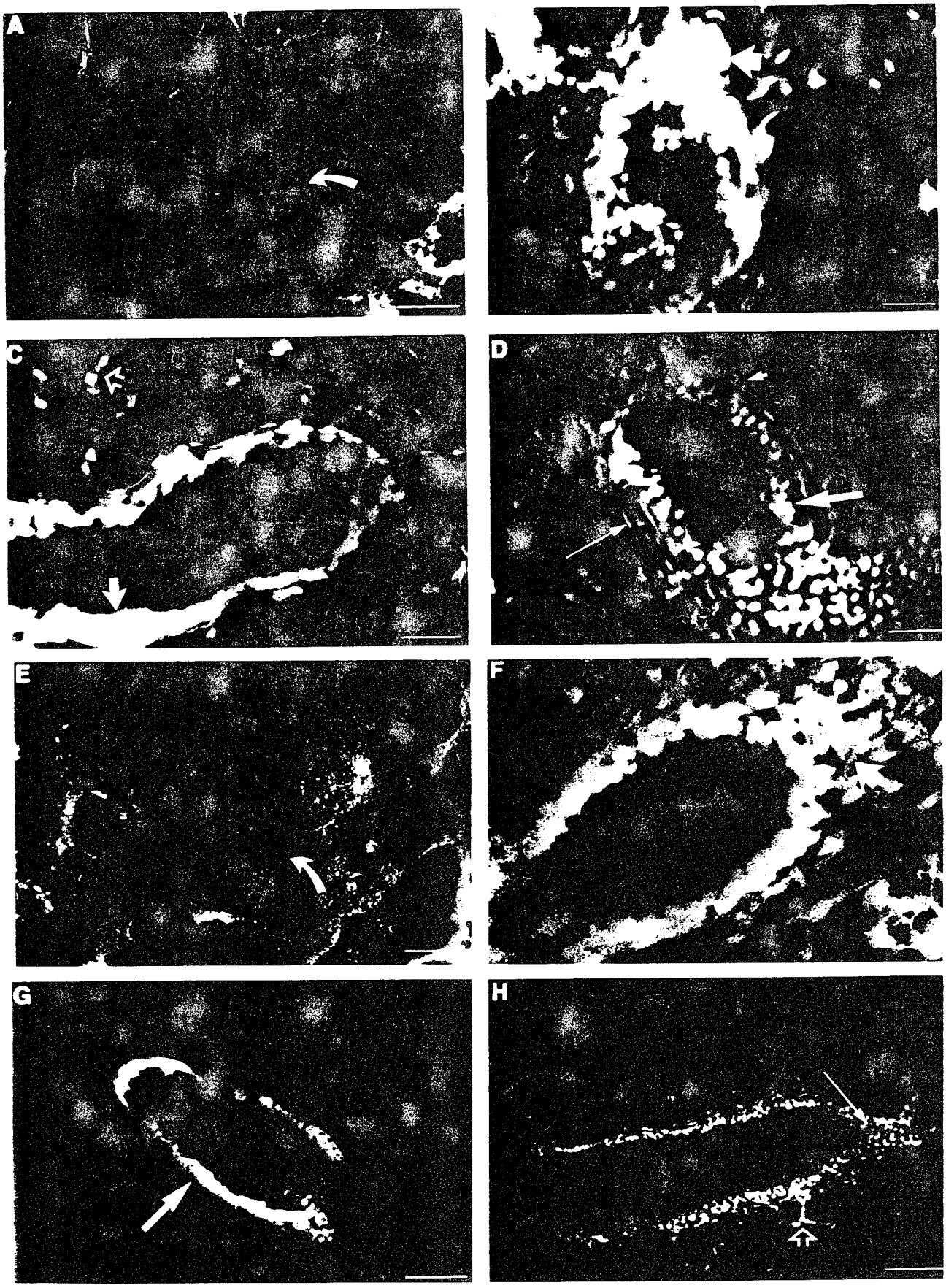
To ascertain whether Ar and Cr-1 are also present in primary cultures of mouse mammary epithelium, 12-week-old mammary epithelium was isolated following collagenase digestion. Cells were seeded onto plastic dishes and cultured without passage and fixed for staining while still sparse. Ar and Cr-1 immunolocalization assays are illustrated in Figure 5A and B. Control primary cultures were treated in the same fashion as tissue sections described earlier (Fig. 5C and D.) In Figure 5A, Ar was consistently detected throughout the epithelial cell population and showed nuclear as well as cytoplasmic localization. Immunoreactive Cr-1 was present in the majority of primary epithelial cells and was largely cytoplasmic with occasional Golgi and nuclear staining (Fig. 5B).

DISCUSSION

Growth factors play a critical role in the proliferation, migration, differentiation, and development in the virgin mouse mammary gland (Daniel and Silberstein, 1987). Detection of Ar mRNA in 4- to 12-week-old mammary gland by RT-PCR and by Western blot analysis indicates a possible role for Ar in ductal proliferation, since other members (TGFα and EGF) are able to stimulate ductal proliferation in ovariectomized mice (Coleman et al., 1988; Vonderhaar, 1987; Snedeker et al., 1991). In these reports, implantation of recombinant EGF or TGFα pellets into regressed mammary glands initiated the reappearance of terminal end bud structures. In addition, Coleman et al. (1988) demonstrated that EGF's activity on regressed end buds was EGF receptor-dependent since EGF receptor levels were 20-fold higher in the proliferative cap cells than in the luminal epithelial cells. The effect of Ar as a mitogen on mammary epithelial cells may be vital for the in vivo growth of mammary epithelial cells since Ar has the ability to induce autophosphorylation and activation of the EGF receptor tyrosine kinase (Johnson et al., 1993). In addition, Ar can stimulate, in an equivalent manner to EGF, the growth of primary human mammary epithelial cells and immortalized human mammary 184A1N4 and MCF-10A cells (Li et al., 1993; Kenney et al., 1993; Normanno et al., 1994).

Ar was localized in the myoepithelial cells, luminal epithelial cells, and cap cells of 4- to 8-week-old virgin mammary gland. Ar was also found in the stroma adjacent to ductal epithelial structures as well as in a minority of preadipocytes and fibroblasts in the fat pad of 4- and 8-week-old virgin glands. Ar is generally expressed by epithelial cells (Li et al., 1992; Kenney et al., 1993; Piepkorn et al., 1994). Localization of Ar protein in the surrounding stroma in the mammary gland does not necessarily indicate that it is sythesized in these cells, since it is a secreted, heparin-binding protein. This wide distribution of Ar in the developing mammary gland may reflect several modes of action in mammary tissue. First, although soluble heparin inhibits the effect of Ar on EGF receptor signaling, Ar-

Fig. 3. Immunofluorescent detection of amphiregulin and cripto-1 in developing postnatal virgin mouse mammary glands for C57BL/6 mice. The affinity purified rabbit polyclonal antiserum stained cells originating in the growing ducts. In 4-, 8-, and 12-week-old glands (Fig. 3B–D), staining for Ar using the anti-peptide antibodies was observed in myoepithelial cells (large arrow), luminal epithelial cells (medium arowhead), cap cells (large arrowhead), adipocytes (open arrowhead), and fibroblasts (thin arrow). The small arrowhead in Figure 3D indicates the end bud tip. For Ar negative controls, staining was abolished by preincubation of the antibody with soluble peptide immunogen representing amino acids 26–44 (Fig. 3A, curved arrows). Staining for Cr-1 in 4-, 8-, and 12-week-old glands (Fig. 3F–H) was observed in myoepithelial cells (large arrow), luminal epithelial cells (thin arrow), cap cells (large arrowhead), and adipocytes (open arrowhead). For Cr-1 negative controls, staining was largely abolished by preincubation of the antibody, with soluble peptide immunogen representing amino acids 97–113 (Fig. 3E, curved arrow). In A–D, bar = 20 μm; in G and H, bar = 50 μm.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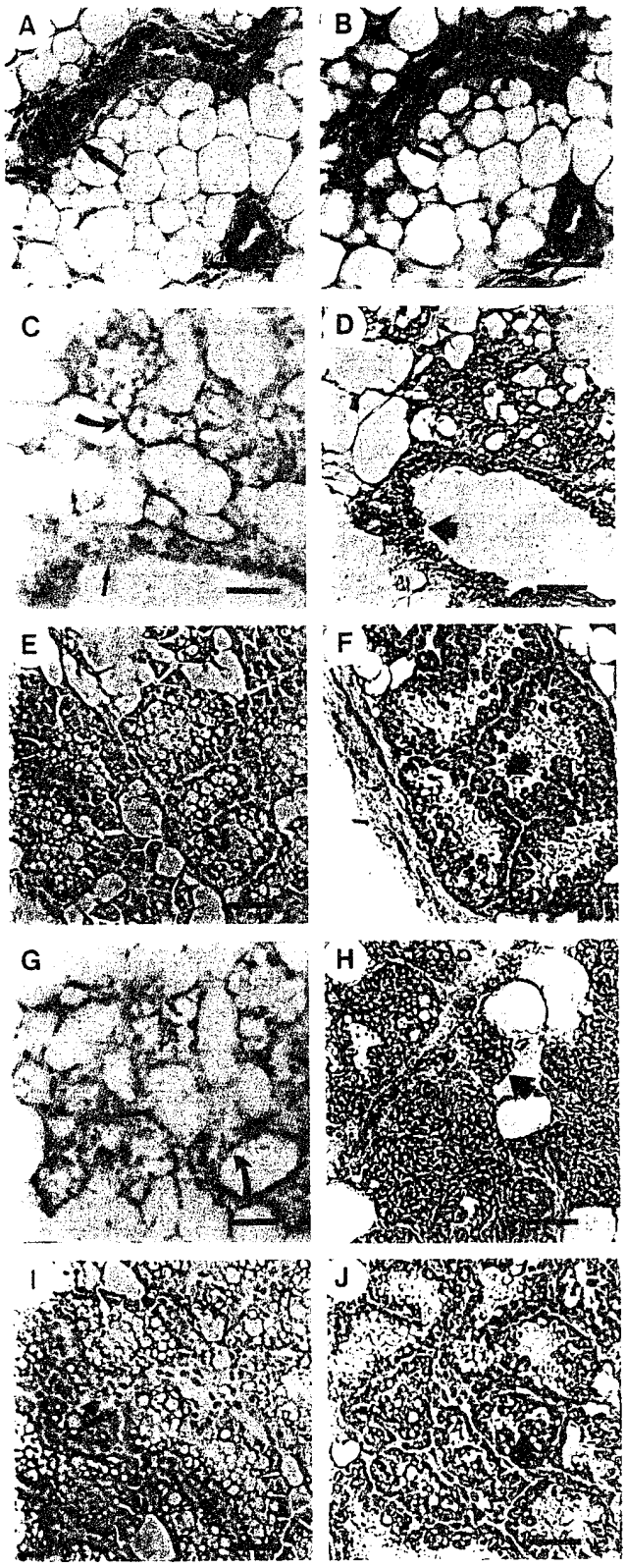

dependent proliferation may require endogenous heparan sulfate-glycosaminoglycans (Li et al., 1993; Piepkorn et al., 1994). Secondly, Ar contains two strongly basic regions in the amino-terminal region that may potentially act as binding sites for heparan sulfate-glycosaminoglycans and as nuclear targeting sequences that are necessary for Ar mitogenic activity (Plowman et al., 1990; Kimura, 1993). Third, the basement membrane surrounding the mammary end bud tip is rich in hyaluronate, whereas the stroma adjacent to the flanks and up to the rear of the end bud is rich in sulfated glycosaminoglycans (Silberstein and Daniel, 1982). Therefore, we can postulate that the involvement of glycosaminoglycans may modulate Ar action in the initiation or maintenance of interductal spacing between the advancing ducts in the growing end buds (Silberstein and Daniel, 1982). Alternatively, sulfated glycosaminoglycans may sequester Ar in the extracellular matrix in a selective and focal fashion to autoinduce the EGF receptor mitogenic pathway in myoepithelial cells that are adjacent to the cap cells (Piepkorn et al., 1994).

Cr-1 mRNA and protein were detected in virgin 4- to 12-week-old, midpregnant, and lactating glands by RT-PCR and Western blot analysis. Several protein species were detected by the anti-CR-1 antibody and that were not detected in the presence of an excess of peptide immunogen. Some of these species were larger than calculated for the Cr-1 translated sequences and could be unknown crossreacting proteins. The human CR-1 protein in GEO colon carcinoma cells is approximately 36 kDa, while the Cr-1 protein expressed in mouse F9 teratocarcinoma cells is 21–24 kDa (Brandt et al., 1994). Since the mouse and human Cr-1 proteins possess at least five potential myristylation sites, two protein kinase C phosphorylation sites, and one protein kinase A phosphorylation site, post-translational modifications as well as additional N-glycosylation of the protein could account for the multiple immunoreactive species that were detected by Western blot analysis and that might vary in a species and/or tissue-specific fashion.

The levels of Ar and Cr-1 were increased two- to threefold during pregnancy and remained high in lactating glands. Brown et al. (1989) and Snedeker et al.

Fig. 4. Immunoperoxidase detection of amphiregulin and Cripto-1 proteins in virgin 5-week-old FVB/N mammary gland and from C57 BL 13.5 day midpregnant and lactating mouse mammary gland sections. Ar and Cr-1 localization in the myoepithelial cells (Fig. 4A and 4B, large arrows) and luminal epithelial cells (Fig. 4A and 4B, medium arrowheads) of the virgin 5-week-old FVB/N mammary gland. Detection of Ar staining for alveolar cells and cell-associated Ar in midpregnant and lactating mammary sections was abolished by preincubation of the antibody with soluble Ar peptide immunogen (Fig. 4C and 4E, curved and small, straight arrows). Cr-1 detection was largely abolished by preincubation of the antibody with soluble Cr-1 peptide immunogen (Fir. 4G and 4I, curved and small, straight arrows). In 13.5 day midpregnant and lactating mammary gland, staining of Ar and Cr-1 proteins were observed in alveolar cells (large arrowhead). Bars represent 50 microns.

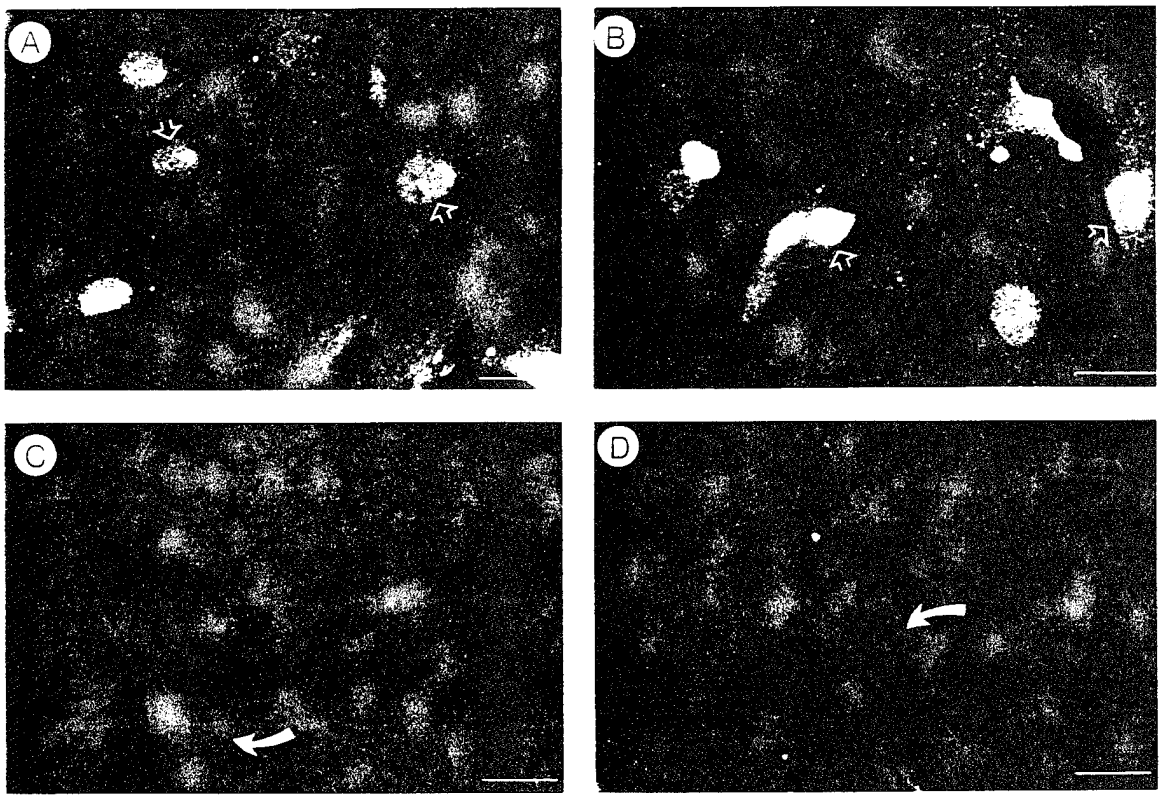

Fig. 5. Amphiregulin and cripto-1 immunostaining in primary mammary cell culture. The affinity purified rabbit polyclonal antisera Ab-2 and 67CR stained cells throughout the entire cellular population (Fig. 5A and B, open arrowheads). Staining for amphiregulin and cripto-1 was abolished by preincubation of the antibodies with soluble peptides amino acids corresponding to 26-44, and amino acids corresponding to 97-113, respectively (Fig. 5C and D, curved arrows). Bar = 20 μm.

(1991) reported that EGF and TFGα could be detected in the virgin midpregnant, and lactating mammary gland and in milk. Liscia et al. (1990) reported the level of TGFα mRNA was also increased two- to threefold in ductal and alveolar cells of pregnant and lactating mammary tissue. Since EGF receptor numbers are upregulated by steroid hormones in sexually mature animals, our results suggest that along with EGF and TGFα, Ar and Cr-1 may account for additional autocrine growth factors that might be necessary for alveolar and lobular development or roles such as upregulation of milk protein production (Haslam et al., 1992). In contrast to Ar and similar to EGF (Snedeker et al., 1991), Cr-1 localization was often observed in the lumen as well as in luminal epithelial, myoepithelial cells, and preadipocytes. Presently, the biological effects of purified recombinant Cr-1 on mammary epithelial cells in vivo is unknown. However, Cr-1 can act as a mitogen on 184A1N4 human mammary eptihelial cells and MDA-MB-453, and SK-Br-3 human breast cancer cells in vitro (Brandt et al., 1994). Since overexpression of human CR-1 has the ability to transform mouse NOG-8 mouse mammary epithelial cells in vitro, Cr-1 may function as a mitogen in vivo in the mammary gland and may also have a similar role in milk.

To date, this is the first report describing the detection and localization of Ar and Cr-1 in the developing mouse mammary gland. Accumulated results suggest that at least four EGF-like family members, TGFα, EGF, Ar, and Cr-1, are expressed during various stages of mouse mammary gland morphogenesis. To further understand the significance of Ar and Cr-1 in mammary development and tumorigenesis, transgenic and gene targeting technologies are required. These experiments are now underway.

ACKNOWLEDGMENTS

We thank Ms. R. Jenkins and the staff of the Animal Facility for their help and cooperation in these studies and to Dr. Cheng-Feng Qi for discussions relating to ICC localizations. We thank all our colleagues for useful discussions and guidance. These studies were supported by a Postdoctoral Minority Award from National Institutes of Health to N.K. and by grants from the Public Health Service, CA54233 and HD 28427 (EDA).

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