Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8417–8421, November 1990
Cell Biology

cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequences

JOHN D. STUBBS*†, CHRISTINE LEKUTIS*, KAREN L. SINGER*, ANHTHU BUI*†, DALE YUZUKI†, USHA SRINIVASAN*, AND GORDON PARRY*‡

*Cell and Molecular Biology Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720; and †Department of Biology, San Francisco State University, CA 94132

Communicated by Melvin Calvin, April 30, 1990 (received for review April 4, 1990)

ABSTRACT **A 2.1-kilobase cDNA coding for a surface protein of mammary epithelial cells has been isolated from a mouse mammary gland λgt11 cDNA library. Sequence analysis of this cDNA reveals an open reading frame of 1389 base pairs that defines a protein with a molecular mass of 51.5 kDa. Structural analysis of the predicted sequence identifies two putative functional domains of the protein: (*i*) an N-terminal cysteine-rich region that is similar to epidermal growth factor-like domains of *Drosophila* Notch-1 protein and (*ii*) a large segment of the sequence that exhibited 54.5% identity with C-terminal domains of human coagulation factors VIII and V. These similarities in structure are used to predict the possible functions of the protein and its means of interaction with the cell surface. mRNA expression was detectable in mammary tissue from nonpregnant animals but was maximal in the lactating gland. In cultured cells, mRNA levels also correlated with the degree of cellular differentiation.**

During the course of lactogenesis, the apical surface of mammary epithelial cells becomes highly specialized to participate in the process of triglyceride secretion into milk (1). Triglycerides are secreted by a unique budding process whereby cytoplasmically formed fat droplets bud from the apical surface and become enveloped by a portion of the apical plasma membrane. The membrane is subsequently secreted with the triglyceride droplet, forming the milk fat globule membrane (MFGM) (1, 2). Studies of the composition of this membrane have been performed and the dominant glycoprotein was shown to be a high molecular mass mucin-like glycoprotein termed polymorphic epithelial mucin (3, 4). This has attracted considerable attention as it is also expressed on a large proportion of human breast tumors (5, 6). Several monoclonal antibodies reactive with this glycoprotein have demonstrated diagnostic potential for monitoring tumor growth and also for imaging tumors (7). cDNA molecules coding for this protein have been cloned and the sequence of the protein has been predicted (8).

Other proteins commonly present in the MFGM include a 155-kDa protein, xanthine oxidase (9), an 80-kDa glycoprotein (10), and glycoproteins with molecular masses in the range of 63–70 kDa (11, 12) and 55 kDa (13). In contrast to the information available on the polymorphic epithelial mucin glycoprotein, little information is available regarding the structure and functions of any of the other MFGM components. Nevertheless, preliminary studies have demonstrated that many of these components may also be differentiation antigens that are expressed on tumor cells (14).

Many previous studies of mammary gland differentiation have demonstrated the utility of the rat and mouse for experimental work and prompted us to choose the mouse for detailed investigations of MFGM glycoproteins that may be important in differentiation and carcinogenesis. In this manuscript, we describe the cloning and sequencing of a cDNA coding for a glycoprotein that is present in the mouse MFGM.§ Analysis of the predicted protein sequence reveals a striking similarity between a region of the protein and epidermal growth factor (EGF)-like domains of *Drosophila* Notch-1 protein. In addition, two other sets of sequence are very similar to regions of clotting factors VIII and V.

EXPERIMENTAL PROCEDURES

Preparation of Mouse MFGM and Generation of Antibodies. Membrane-encased milk fat globules were prepared from freshly collected milk by flotation centrifugation. The membranes were subsequently released by freeze–thaw fragmentation and collected by centrifugation at 100,000 × *g* for 1 hr. Approximately 500 μg of membrane protein plus Freund's adjuvant was used to immunize New Zealand white rabbits at monthly intervals and serum was collected 10 days after the fourth immunization.

Construction and Screening of a λgt11 Mammary cDNA Library. RNA was isolated from lactating mouse mammary glands by the method of Chirgwin *et al.* (15) and cDNA was prepared using $(dT)_{12-18}$ (Pharmacia LKB) as primer and enzymes and reagents from a cDNA cloning kit (Amersham). The fraction of cDNA >500 base pairs (bp) was ligated into the *Eco*RI site of λgt11 arms, packaged into λ coats, and plated on *Escherichia coli* Y1090r⁻ (Promega). Approximately 5×10^5 recombinants were screened on nitrocellulose filter lifts with an affinity-purified immunoglobulin fraction from the antisera raised against the purified mouse MFGM (see *Results* for details of affinity purification). A positive clone with a 2.1-kilobase (kb) insert was selected for further characterization.

The λgt11 phage stock containing the 2.1-kb insert was used to infect *E. coli* Y1089r⁻(Promega) and lysogenic colonies were selected. β-Galactosidase fusion protein synthesis was induced with isopropyl β-D-thiogalactoside (10 mM), and, after SDS/PAGE of bacterial proteins, fusion protein was detected on Western blots.

DNA Sequence Analysis. The 2.1-kb cDNA *Eco*RI insert was ligated into $pT_7/T_3\alpha$-18 and $pT_7/T_3\alpha$-19 and transformed into competent *E. coli* DH5α (Bethesda Research Laboratories). In addition, the insert was ligated into M13mp18, replicative form (Pharmacia LKB), and used to transform *E. coli* JM101. The dideoxynucleotide chain-termination method (16) was used with T7 DNA polymerase (United States Biochemical) to sequence the insert by both a double-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38337).

stranded denatured technique with the pT_7/T_3 vectors and, independently, by single-stranded sequencing of DNA isolated from M13mp18 recombinant phage (17).

Northern Blot Analysis. ^{32}P-radiolabeled cDNA probe was prepared by random-primer labeling of the isolated 2.1-kb insert DNA using [^{32}P]dCTP (3000 Ci/mmol, 1 Ci = 37 GBq; Amersham). RNA was isolated using guanidine isothiocyanate (15), resolved on formaldehyde/agarose gels, and transferred to nylon membranes (Hybond-N, Amersham). Blots were probed and washed as described by Streuli and Bissell (18).

Peptide Synthesis and Production of Antibodies. Peptides were synthesized at the Microchemical Facility at the University of California, Berkeley. Unconjugated peptide (600 μg; 22 amino acids long) in Freund's complete adjuvant was injected subcutaneously into rabbits, followed by repeated immunizations 3 and 5 weeks later. Rabbits were bled 10 days after the third injection.

Computer Analyses. DNA sequence analysis and searches of GenBank and National Biomedical Research Foundation Protein Identification Resource were performed using a software package from the University of California, San Francisco computing facility and programs of the Bionet National Computer Resource for Molecular Biology (search date, November 1989). Data base searches used a program devised by Pearson and Lipman (19). Sequence alignments were performed using MALIGN and GENALIGN programs (20).

RESULTS

Our approach to obtaining structural information about glycoproteins of the MFGM was to first identify the principal components of the mouse membrane and subsequently to generate antisera reactive with the major components other than the well-characterized glycoprotein polymorphic epithelial mucin. The antibodies would then be used to screen cDNA expression libraries made from lactating mammary gland mRNA, and the isolated cDNAs were then sequenced. Protein sequence would then be predicted on the basis of the cDNA sequence.

Fat globules were isolated from mouse milk by differential centrifugation. Resolution of the solubilized components by SDS/PAGE revealed the presence of four major Coomassie blue-staining bands with molecular masses of 53, 66, 155, and 220 kDa (Fig. 1*A*). The dominance of 66-kDa species prompted us to focus our efforts on these components. Extensive efforts to generate monoclonal antisera recognizing the 66-kDa components were unsuccessful and it became necessary to develop an affinity-purified polyclonal antiserum as an alternative. Antisera were first raised in rabbits using purified membranes as immunogens. The resulting antisera were subsequently purified by absorption against 66-kDa antigens immobilized on nitrocellulose, as described by Smith and Fisher (21). The specificity of these antibodies is shown in Fig. 1*B*. Strikingly, this affinity-purified antibody recognized not only the 66-kDa band but additionally a lower molecular mass form at 53–55 kDa, prompting us to refer to the antigen as component 66/53 kDa.

The antibody preparation was then used to screen the unamplified λgt11 library, and a phage plaque was isolated. *Eco*RI restriction of the purified phage DNA revealed the insert to be 2.1 kb long. When the β-galactosidase fusion protein was expressed in the bacterial strain Y1089 under lysogenic conditions, fusion proteins from 170 kDa to 40 kDa were identified (Fig. 1*C*). The range of bands observed on the gel probably reflects degradation of the fusion protein. If the largest band observed corresponds to the intact fusion protein, then, based on the difference in its size and that of the β-galactosidase protein, it was estimated that the protein

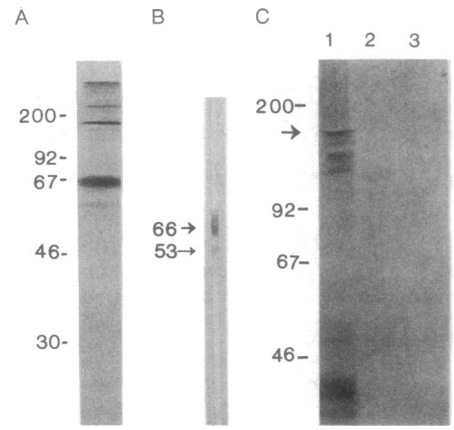

FIG. 1. (*A*) Major components of mouse MFGMs. Purified mouse MFGM components were resolved by SDS/PAGE on a 10% polyacrylamide gel and then stained with Coomassie blue. The major components migrated with molecular masses of 220, 155, 66, and 53 kDa. (*B*) Specificity of the affinity-purified antibody used for screening the expression library. Western blot of MFGM proteins resolved on a 7.5% polyacrylamide gel was probed with the affinity-purified anti-66 kDa. The antibody reacted with a 66-kDa band and also to a lesser extent with a 53-kDa component. (*C*) Identification of β-galactosidase fusion proteins produced by Y1089 λgt11-infected bacteria. Proteins were resolved by SDS/PAGE, and a Western blot was then probed with anti-66 kDa exhibiting the specificity shown in *B*. Lanes: 1, a λgt11 construct with the 2.1-kb insert, selected from the library using anti-66 kDa; 2, a λgt11 construct with a 400-bp insert coding for a heparan sulfate proteoglycan core protein; 3, a λgt11 vector with no insert.

coded for by the 2.1-kb DNA had a molecular mass of ≈54 kDa.

Nucleotide Sequence Analysis of the 2.1-kb Insert. The complete sequence of the 2.1-kb cDNA is shown in Fig. 2, as is the predicted amino acid sequence. Characteristic features of the sequence are a 3′ poly(A) tail and an untranslated 3′ region of 658 nucleotides. Significantly, the usual consensus sequence of AATAAA, specifying polyadenylylation was not found but the sequence AATACA is located 17 nucleotides from the poly(A) tail and possibly serves as an alternate polyadenylylation signal. At the 5′ end of the cDNA, two possible ATG start codons are found with the first codon preceded by the sequence GCAGC, which is frequently associated with ATG start codons (22).

Analysis of the Predicted Protein Sequence. The identity of the protein coded for by the 2.1-kb cDNA as the 66/53-kDa MFGM protein was confirmed by N-terminal sequence analysis of 66- and 53-kDa proteins isolated from SDS/PAGE-resolved MFGM preparations. Although the N-terminal sequence of the 66-kDa protein was blocked, that of the 53-kDa protein was shown to be: Ala-Ser-Gly-Asp-Phe-Xaa-Asp-Ser-Ser-Leu-Xaa-Leu-Asp-, which corresponded to the sequence 21 amino acids beyond the methionine start codon. Confirming evidence was obtained by raising an antibody against the predicted sequence of amino acids 389–410 and demonstrating that the antibody recognized 66-kDa and 53-kDa MFGM proteins (data not shown).

Hydropathicity plots revealed the first 21 amino acids to be a typical signal sequence and to be the only significant stretch of hydrophobic sequences in the molecule. The N-terminal 86 amino acids beyond the signal sequence contain a total of 12 cysteine residues, constituting a cysteine-rich domain of the molecule. The spacing of the cysteine residues is noteworthy, in that a Cys-Xaa$_4$-Cys-Xaa$_5$-Cys-Xaa$_n$-Cys-Xaa-Cys-Xaa$_8$-Cys tandem repeat constitutes the bulk of the cysteine-rich domain. Adjacent to this cysteine-rich domain is a short sequence containing five closely positioned proline residues.

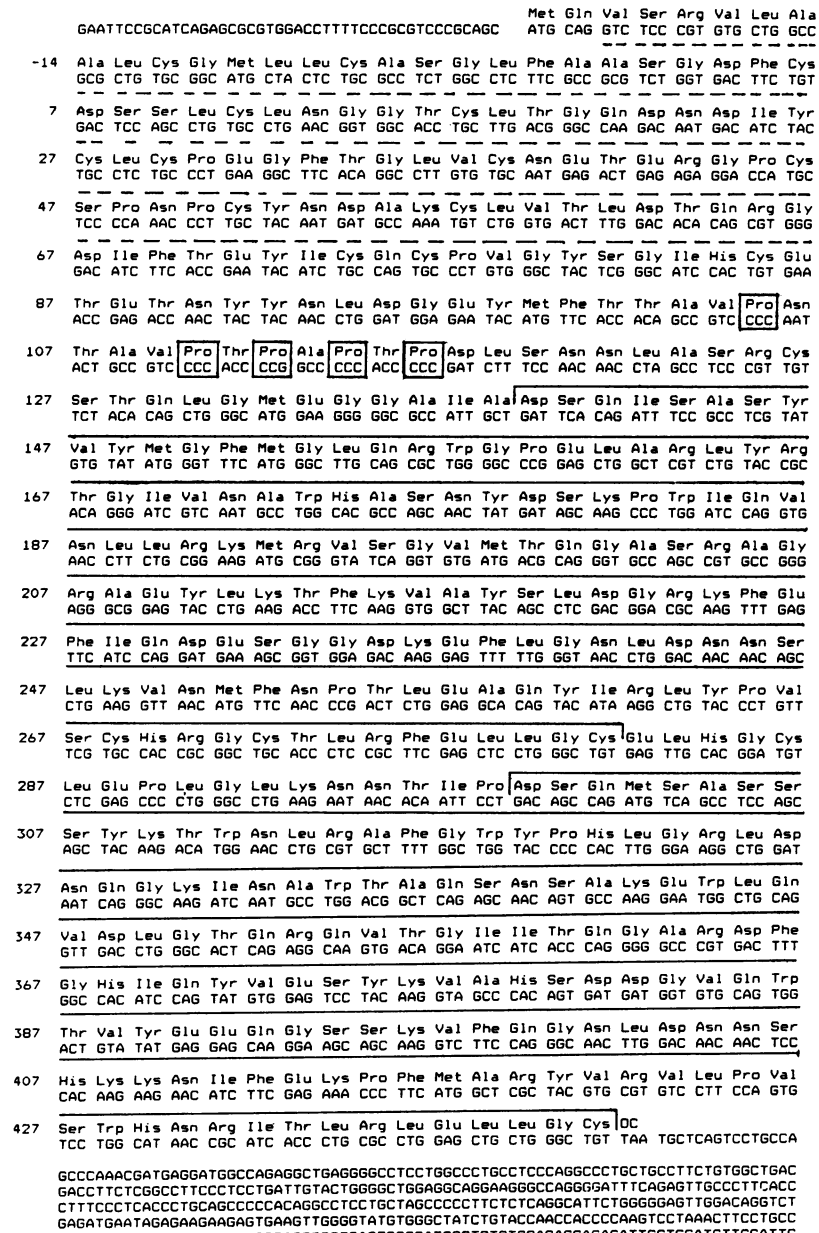

FIG. 2. Nucleotide sequence and predicted amino acid sequence of the 2.1-kb cDNA coding for the 66/53-kDa MFGM protein. The translated sequence is a protein with a molecular weight of 51,473. Dashed line, cysteine-rich region of the molecule; solid line, related internally repeated sequences involving amino acids 139–281 and 299–441; boxes, proline-rich region.

This is followed by a tandemly repeated sequence, comprising amino acids 139–281 and 299–441 (Fig. 2).

The nucleotide and amino acid sequences of the MFGM protein were compared with sequences found in the Genbank, National Biomedical Research Foundation, and Swiss-Prot data bases. The C-terminal region of the MFGM protein sequence was found to be very similar to the C-terminal sequence of human blood clotting factors VIII and V. Identity at a nucleotide level was found to be 54.2% in an 866-nucleotide overlap, and identity at a protein sequence level was determined to be 42% in a 320-amino acid overlap (Fig. 3). Factors VIII and V have been shown (32) to share closely related C-terminal domains and it is these homologous sequences that are closely related to the MFGM protein.

Strong similarity of the cysteine-rich domain of component 66/53 kD to other sequences in the data bases was also found. The most striking similarity was discovered with the *Drosophila* protein Notch-1 (23), as well as with neurogenic repetitive locus proteins, 95F and delta (24, 25). The same sequence was also very similar to a cysteine-rich region of a proteoglycan core protein (26) (Fig. 4). Aside from the cysteine spacing pattern, there is also close similarity of intervening amino acid sequences, a feature that also applies to the proteins EGF, transforming growth factor α, and lin-12 homeotic protein, which have only a single cysteine unit with this or a closely related spacing (27–30).

Regulation of Expression of mRNAs Coding for the MFGM Protein. The 2.1-kDa cDNA isolated was used to probe mRNA levels in mammary tissue and in cultured mammary epithelial cells. The cDNA identified a mRNA molecule that was 2.1 kb long, confirming that the cDNA represented a full-length copy of the mRNA (Fig. 5). Northern blot analysis revealed that the mRNA was relatively abundant in mammary tissue from lactating mice and was also expressed in

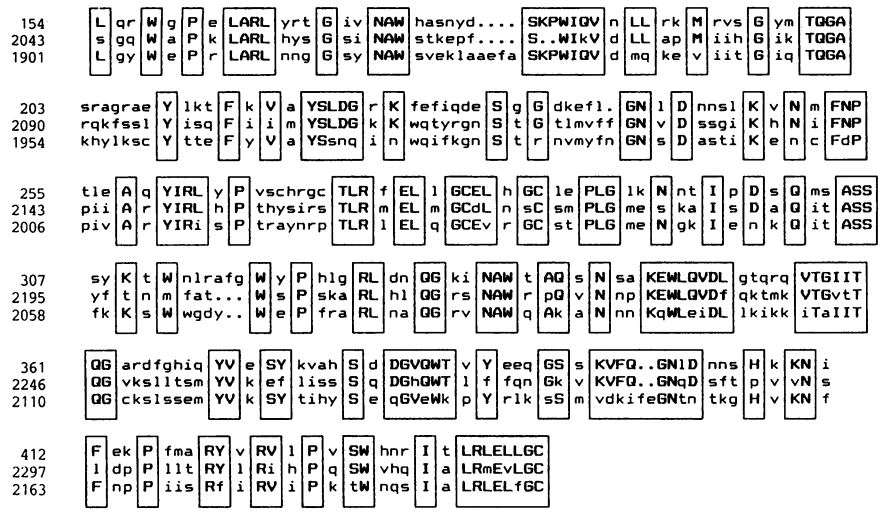

FIG. 3. Comparison of the amino acid sequences of human coagulation factors VIII and V with the predicted sequence of the 66/53-kDa protein. The sequences were aligned using the MALIGN program. Dots in the sequence refer to gaps that were inserted for optimal alignment of the three sequences. The homologous regions of factors VIII and V are the C-terminal domains of the molecules.

mammary glands of virgin mice at a reduced level. Expression of the mRNA in cultured cells was also examined. Cells isolated from late pregnant mice retain their differentiated characteristics best in culture when maintained on collagen gels that are made to float in the culture medium (31). Cells on attached collagen gels or on tissue culture plastic dishes are unable to express tissue specific mRNAs to the same extent. Maximum expression of mRNA for the MFGM protein was observed in cells on floating collagen gels but cells on plastic expressed significantly reduced quantities of mRNA (Fig. 5).

DISCUSSION

We are attempting to understand structural and functional features of glycoproteins on mammary epithelial cell surfaces by cloning and sequencing cDNAs for the major membrane proteins. In this paper, we present data on a cDNA for the 66/53-kDa membrane protein. The cDNA is 2086 bp long and carries coding information for the full-length mRNA. An open reading frame of 1389 bp has been identified that is flanked by a 39-bp 5′ leader and a 658-bp 3′ untranslated region of unknown function. The primary translation product defined by the open reading frame has a molecular weight of 51,473. Structural analysis of this amino acid sequence identifies five structural domains of the molecule: (*i*) a terminal 22-amino acid sequence that has characteristics of a signal sequence, (*ii*) an 86-amino acid cysteine-rich sequence that begins with the N-terminal sequence of the mature protein, (*iii*) a short stretch of amino acids 87–121 that includes a cluster of aromatic amino acids followed by a series of proline residues, (*iv*) two domains, each of 142 amino acids, that are very similar to each other. As the signal sequence is the only hydrophobic sequence in the whole molecule and this is cleaved (at least in the 55-kDa form), we hypothesize that the protein is a peripheral membrane protein. The difference in size between the predicted translated molecular mass of 51.5 kDa and the observed sizes of 55 and 66 kDa presumably reflect glycosylation at the many potential O- and N-linked glycosylation sites.

A striking feature of the predicted protein sequence is its similarity to regions of other proteins. Nearly 70% of the sequence is very similar to the C-terminal C1 and C2 regions of factors V and VIII. Although the function of these domains of the clotting factors remains to be fully established, they

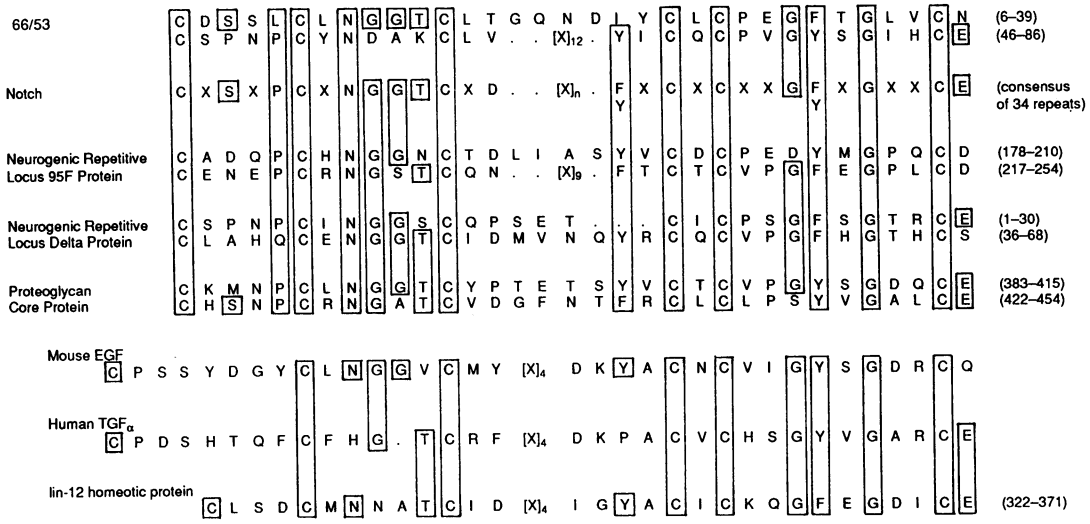

FIG. 4. Comparison of the cysteine-rich sequence (amino acids 6–86) of the 66/53-kDa protein with EGF-like regions of *Drosophila* Notch-1, neurogenic repetitive locus 95F and delta proteins, a human proteoglycan core protein, mouse EGF, human transforming growth factor α, and *Caenorhabditis elegans* lin-12 homeotic protein. The first four proteins containing tandem repeated units with cysteines spaced according to the pattern: Cys-Xaa$_4$-Cys-Xaa$_5$-Cys-Xaa$_n$-Cya-Xaa-Cys-Xaa$_8$-Cys. Several of the amino acids in the intervening sequences are also identical. Notch-1 has 34 repeated segments and most show homology to the 66/53-kDa protein. The sequence shown is a consensus of the 34 repeats as described by Wharton *et al.* (23). Mouse EGF, human transforming growth factor α, and lin-12 homeotic protein have a single cysteine unit with similar (though not identical) spacing to the 66/53-kDa protein.

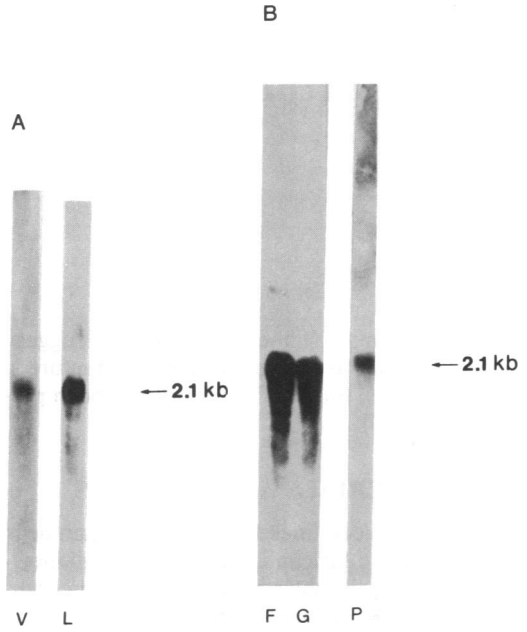

FIG. 5. Northern blots of RNA from virgin (lane V) and lactating (lane L) mammary tissue (*A*) and of RNA from primary cultures of cells maintained on plastic tissue culture dishes (lane P) or on attached (lane G) or floating collagen gels (lane F) (*B*). Blots were probed using the 2.1-kb cDNA probe coding for the 66/53-kDa protein.

compose part of a larger functional domain that promotes binding to anionic phospholipids on the surfaces of platelets and endothelial cells (ref. 32 and references therein). It is conceivable, then, that the C-terminal repeat segments of the 66/53-kDa protein are involved in phospholipid binding, possibly as a means of attaching to the membrane surface. The similarity of the cysteine-rich domain to Notch-1 and other EGF-like sequences was also highly significant. Although EGF has been shown to promote growth and differentiation of several cells and tissues, the function of EGF-like domains in other proteins is not yet known. One biochemical activity associated with an EGF-like domain is that of calcium binding, as identified in the vitamin K-dependent coagulation proteins (33). However, the EGF-like domains of component 66/53 kDa do not contain the consensus of amino acids associated with calcium binding.

Studies of component 66/53 kDa mRNA levels in virgin and lactating mice revealed that mRNA expression was developmentally regulated. Expression in cultured epithelial cells was modulated by the culture substratum and was maximal when conditions to promote differentiation were used. These *in vivo* and *in vitro* studies then establish this protein as a cell surface differentiation marker of mammary epithelia.

The MFGM protein we have identified, to our knowledge, appears to be unrelated to any characterized MFGM component. The major protein in the molecular mass range of 65–70 kDa in MFGM from bovine and guinea pig is butyrophilin, a relatively insoluble transmembrane protein. Protein 66/53 kDa appears to be distinct from butyrophilin (34). Another glycoprotein, gp70, has been identified on human MFGMs and shown to be expressed on many breast carcinomas but no information is available concerning its structure (12). A 55-kDa glycoprotein termed gp55 has also been identified in guinea pig membranes (13). This is similar to the 66/53-kDa protein in that it is a peripheral membrane protein but, in contrast to protein 66/53 kDa, no higher molecular mass forms of gp55 have been found. Although a function for component 66/53-kDa is not known, we believe it is appropriate to name this protein and refer to it as *m*ilk *f*at *g*lobule-*E*GF factor *8* (MFG-E8).

We thank Drs. I. Mather, T. Keenan, and W. Henzel for providing us with N-terminal sequence data. The research was supported by the Department of Energy, Office of Health Effects Research under Contract DEAC03-76SF 00098 and by National Institutes of Health Grant RR05918.

1. Mather, I. H. (1987) in *The Mammary Gland*, eds. Neville, M. C. & Daniel, C. W. (Plenum, New York), pp. 217–267.
2. Stemberger, B. H., Walsh, R. M. & Patton, S. (1984) *Cell Tissue Res.* **236**, 471–475.
3. Shimizu, M. & Yamauchi, K. (1982) *J. Biochem. (Tokyo)* **91**, 515–524.
4. Johnson, V. G., Greenwalt, D. E., Heid, H. W., Mather, I. H. & Madara, P. J. (1985) *Eur. J. Biochem.* **151**, 237–244.
5. Taylor-Papadimitriou, J., Peterson, J. A., Arklie, J., Burchell, J., Ceriani, R. L. & Bodmer, W. F. (1981) *Int. J. Cancer* **28**, 17–21.
6. Kufe, D., Inghirami, G., Abe, M., Hayes, J., Justi-Wheeler, H. & Schlom, J. (1984) *Hybridoma* **3**, 223–232.
7. Epenetos, A. A., Canti, G., Taylor-Papadimitriou, J., Curling, M. & Bodmer, W. F. (1982) *Lancet* **ii**, 1004–1006.
8. Gendler, S., Taylor-Papadimitriou, J., Duhig, T., Rothbard, J. & Burchell, J. (1988) *J. Biol. Chem.* **263**, 12820–12833.
9. Bruder, G., Heid, H. W., Jarash, E. D. & Mather, I. H. (1983) *Differentiation* **23**, 218–225.
10. Greenwalt, D. E. & Mather, I. H. (1985) *J. Cell Biol.* **100**, 397–408.
11. Franke, W. W., Heid, H. W., Grund, C., Winter, S., Freudenstein, C., Schmid, E., Jarash, E. D. & Keenan, T. W. (1981) *J. Cell Biol.* **89**, 485–494.
12. Imam, A., Laurence, D. J. R. & Neville, A. M. (1981) *Biochem. J.* **193**, 47–54.
13. Mather, I. H., Bruder, G., Jarash, E. D., Heid, H. W. & Johnson, V. G. (1984) *Exp. Cell Res.* **151**, 277–282.
14. Ceriani, R. L., Sasaki, M., Sussman, H., Wara, W. M. & Blank, E. W. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5420–5424.
15. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
17. Toneguzzo, F., Glynn, S., Levi, E., Mjolsness, S. & Haday, A. (1988) *Biotechniques* **6**, 460–469.
18. Streuli, C. H. & Bissell, M. J. (1990) *J. Cell Biol.* **110**, 1405–1415.
19. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
20. Sobel, E. & Martinez, H. M. (1986) *Nucleic Acids Res.* **14**, 363–374.
21. Smith, D. E. & Fisher, P. A. (1984) *J. Cell Biol.* **99**, 20–28.
22. Kozak, M. (1984) *Nucleic Acids Res.* **12**, 857–872.
23. Wharton, K. A., Johansen, K. M., Xu, T. & Artavanis-Tsakonas, S. (1985) *Cell* **43**, 567–581.
24. Knust, K., Dietrich, U., Tepass, U., Bremer, K. A., Weigel, D., Vassin, H. & Campos-Ortega, J. A. (1987) *EMBO J.* **6**, 761–766.
25. Vaessin, H., Bremer, K. A., Knust, E. & Campos-Ortega, J. A. (1987) *EMBO J.* **6**, 3431–3440.
26. Krusius, T., Gehlsen, K. R. & Ruoslahti, E. (1987) *J. Biol. Chem.* **262**, 13120–13125.
27. Greenwald, I. (1985) *Cell* **43**, 583–590.
28. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y. & Coeddel, D. V. (1984) *Cell* **38**, 287–297.
29. Gray, A., Dull, T. J. & Ullrich, A. (1983) *Nature (London)* **303**, 722–725.
30. Doolittle, R. F., Feng, D. F. & Johnson, M. S. (1984) *Nature (London)* **307**, 558–560.
31. Parry, G., Cullen, B., Kaetzel, C. S., Kramer, R. & Moss, L. M. (1987) *J. Cell Biol.* **105**, 2043–2051.
32. Kane, W. H. & Davie, E. W. (1988) *Blood* **71**, 539–555.
33. Handford, P. A., Baron, M., Mayhew, M., Willis, A., Beeseley, T., Brownlee, G. G. & Campbell, I. D. (1990) *EMBO J.* **9**, 475–480.
34. Jack, L. J. W. & Mather, I. H. (1990) *J. Biol. Chem.* **265**, 14481–14486.