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THE PRODUCT OF THE HUMAN MUC1 GENE WHEN SECRETED BY MOUSE CELLS TRANSFECTED WITH THE FULL-LENGTH cDNA LACKS THE CYTOPLASMIC TAIL

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SUMMARY: The polymorphic epithelial mucin (PEM) is found as a cell associated transmembrane protein with an extracellular domain made up largely of tandem repeats and also as a soluble form in some body fluids and culture supernatants. To determine whether the soluble form can arise without the mechanism of alternative splicing mouse cells have been transfected with an expression construct containing the full-length cDNA, and the supernatants of the transfectants analyzed for the presence of the mucin. The presence of mucin in the supernatants could indeed be detected in a radioimmunoassay and by immunoprecipitation using monoclonal antibodies to the tandem repeat region of the core protein, indicating that release of the soluble form can occur without alternative splicing. The soluble form was not however precipitated with a polyclonal antiserum to the cytoplasmic tail, suggesting that it was released from the membrane by the action of a protease.

The human MUC1 gene codes for a mucin glycoprotein (the polymorphic epithelial mucin or PEM) which is expressed by most glandular epithelia and which shows increased expression in the breast at lactation and in malignancy (1). The nucleotide sequence of the cDNA has been elucidated and the predicted amino acid sequence of the core protein shows it to be a transmembrane protein, with an extracellular domain made up largely of repeats of 20 amino acids, and a cytoplasmic tail of 69 amino acids (3-5). The number of tandem repeats varies with the individual allele, making this gene an expressed VNTR (variable number of tandem repeats) (6). Using antibodies reactive with epitopes in the tandem repeat domain of the core protein, the MUC1 product has also been detected in milk and in the serum of some breast and ovarian cancer patients (7-9). Furthermore, some breast cancer cell lines have been found to secrete

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the mucin into the supernatant (10), as well as expressing it as a transmembrane protein.

The mechanism by which the mucin is released as a secreted product remains unclear. However, it has been suggested that in the case of the breast cancer cell line, T47D, this involves an alternative splicing event, whereby a stop codon (normally present in an intron) is introduced into the sequence before the transmembrane and cytoplasmic domains (10). We have used mouse mammary epithelial cells, transfected with the full-length human MUC1 cDNA to analyze the secreted mucin, where alternative splicing is not a possibility. Using a monoclonal antibody to the extracellular domain, and a polyclonal antiserum to the cytoplasmic tail (11), we show that in the mucin found in the supernatant of the transfectants, the cytoplasmic tail is absent. Our results indicate that mucin can be secreted without the mechanism of alternative splicing and suggest that PEM may be released from the cell surface by some form of protease activity.

MATERIALS AND METHODS

Cells: The mouse mammary epithelial tumour cell line 410.4 was originally isolated from a single spontaneously arising mammary tumour of a BALB/cf C3H mouse (12) and was kindly provided by Dr. Bonnie Miller (Michigan Cancer Foundation, Detroit, MI). The MCF-7 cell line developed by Soule and colleagues (13) was obtained from the same Institute. All cell lines were cultured in Dulbecco's modified Eagles medium, supplemented with 10% foetal calf serum.

Antibodies: The antibodies HMFG-1, HMFG-2 and SM-3 are directed to core protein epitopes found in the tandem repeat domain of PEM (14-16). CT1 is a polyclonal antibody raised against a peptide comprising the terminal 17 amino acids of the mucin gene product (11).

Plasmids: Full length cDNA of 3762bp from the MUC1 gene was cloned into the pCMV5 mammalian expression vector (17) kindly supplied by Dr. D.W. Russell (University of Dallas) as previously described (2). This vector contains the SV40 origin, the cytomegalovirus major immediate early gene promoter and the human growth hormone fragment with transcriptional termination and polyadenylation signals. The plasmid will be referred to as pCMV5-PEM. The plasmid pSV2neo (18) was used for introduction of a selectable marker.

Calcium Phosphate Mediated Transfection: Subconfluent 410.4 cells were trypsinised and seeded at 5×10^5 cells (in 10ml of DMEM with 10% FCS) per 90mm dish (Nunc) 12-18 hr prior to transfection. One to two hr before transfection, medium was aspirated and fresh medium was added. The cells were then transfected with plasmid DNA prepared by standard methods. 10µg of cDNA construct and 2µg of the plasmid pSV2neo containing the neomycin resistance gene were co-precipitated with calcium phosphate at pH 7.05 (19). Control plates were transfected with 10µg of herring sperm DNA (Sigma) and 2µg of pSV2neo plasmid precipitated in the same way. After 5-6 hr at 37°C dishes were washed with serum free medium, until no trace of a precipitate could be seen under a microscope, and refed with warm DMEM containing 10% FCS. After 48 hr medium was aspirated and fresh medium containing

G418 (starting at 100µg/ml and increasing the concentration every 48 hr to a maximum concentration of 800 µg/ml) was added. Medium was changed every 3-4 days for the next 4 weeks until growing colonies could be ring cloned and expanded.

Radioimmunoassay: The radioimmunoassay was a modified version of that described by Burchell et al. (7). Briefly, 5µg of purified HMFG-2 antibody were dried on to individual wells by incubating at 37°C overnight. Before use, the plate was blocked for 1 hr with 5% BSA in PBSA containing 0.05% Tween, and then washed 5 x with PBSA/Tween 0.05% 50µl of test supernatants plus suitable negative controls were then added to individual wells and incubated for 2 hr at room temperature. After washing as above the iodinated antibody (HMFG-1 or HMFG-2, 400,000 cpm/well in 50µl of PBSA) was added and incubated for 2 hr at room temperature. The washing step was repeated as above and individual wells were then cut and counted.

FACS Analysis: Confluent cultures of cells were trypsinised and resuspended in 2% FCS in PBSA (protein buffer). Cells were then syringed through a 25G needle three times, to achieve a single cell suspension, and 1ml aliquots containing 10^5 cells were dispensed into 20ml Universal tubes. Samples of cells were then incubated with the antibodies HMFG-1, HMFG-2 and SM-3 and bound antibody detected with FITC conjugated rabbit anti mouse immunoglobulin (DAKO). Controls were set up simultaneously in which the first antibody was replaced by protein buffer. 10,000 or 5,000 cells were analysed in a fluorescence-activated cell sorter FACS IV (Becton Dickinson).

Immunoprecipitation of labelled cells and Supernatant: Cells were grown for 24 hr in 6 cm dishes to 70-80% confluence and starved for 2 hr prior to labelling in DMEM with 10% of the usual amount of glucose (0.45g/l). Labelling took place for 16 hr in 0.5 ml of the above medium containing 100µCi of D-[6-^{3}H] Glucosamine Hydrochloride, (Amersham TRK 398, specific activity 26 Ci/mmol.). Supernatant was harvested, and after washing in ice cold PBS, attached cells were lysed in 400µl of lysis buffer: the same quantity of lysis buffer was also added to 1 ml of the labelled supernatant after centrifuging to remove any cells. Lysis buffer contained 40mM sodium phosphate pH 7.2, 250mM NaCl, 50mM sodium fluoride, 5mM EDTA, 1% Triton X-100, 1% deoxycholate, 25µg/ml leupeptin, 100µg/ml PMSF, 10mM benzamidine and 10µg/ml aprotinin.

Immunoprecipitation was by a standard method (20) using 20 µl of neat CT1 antisera or 100 µl of HMFG-1 monoclonal antibody tissue culture supernatant. Complexes were collected on Protein A Agarose (Sigma) and sequentially washed 4 times (1 ml each wash) in (1) lysis buffer (2) 0.5 M NaCl in lysis buffer, (3) lysis buffer, and (4) millipore water. The complexes were resuspended in 50 µl of 2X sample buffer (21), heated at 95°C for 5 mins and the Protein A Agarose was removed by centrifugation. Samples were electrophoresed on a 5% PAGE gel (3% stacking gel) and visualized by fluorography with Amplify (Amersham International).

RESULTS

Isolation and characterisation of mouse cell lines expressing the MUC1 gene: The development of the pCMV5PEM expression construct containing the full length MUC1 cDNA under the control of the CMV promoter has already been described (2). Since the MUC1 gene is normally expressed in epithelial cells, a mouse mammary epithelial cell line, 410.4 (12)

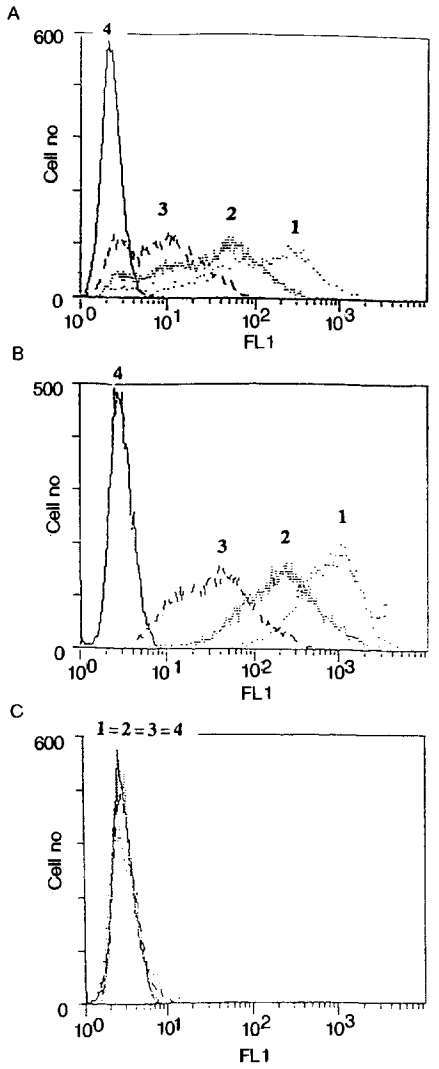

FIGURE 1

Surface binding of anti-mucin antibodies to cell lines detected by FACS analysis. Antibodies HMFG-1, HMFG-2 and SM-3 were bound to A. CDE 2-1, B. CDE 2-3, and C. CDE 1-1 control cells and bound antibody detected by incubation with fluorescein labelled goat anti-mouse immunoglobulin followed by FACS analysis. 1 = HMFG-1, 2 = HMFG-2, 3 = SM-3 and 4 = control (protein buffer replaced the first antibody).

was chosen as recipient for transfection. Transfection was as described in the Materials and Methods and the pCMV5-PEM plasmid was co-transfected with a plasmid containing the neomycin resistance gene. After selection with G418, single colonies were picked and expanded for analysis. A control transfectant (CDE1-1) expressing only the neomycin resistance plasmid was also isolated. Two clones were isolated from the 410.4 cell line transfected with the MUC1 cDNA, referred to as CDE2-1 and CDE2-3, and expression of the mucin was confirmed by demonstrating positive immunohistochemical staining with

three anti-mucin antibodies HMFG-1, HMFG-2 and SM-3. The antibodies react with the tandem repeat region of the core protein of the breast mucin which is found in the extracellular domain of the cell associated mucin. They show differential activity with the normal versus the cancer associated mucin (15), the most striking difference being seen with SM-3 which does not react with the lactating breast but reacts with 92% of breast cancers (22).

To quantitate mucin epitope expression on the surface of the transfectants, live cells were stained with the three anti-mucin antibodies followed by fluorescent labelled goat anti-mouse immunoglobulin and analysed in a fluorescence activated cell sorter (FACS). Figure 1 shows the profile of reactivity of each antibody and it can be seen that while the level of expression in each case is quite heterogeneous there is a hierarchy of HMFG-1 > HMFG-2 > SM-3.

Characterization of PEM in supernatants from the transfectant: The presence of the MUC1 product in the supernatant of the transfectants was then confirmed using a sandwich radioimmunoassay, and the antibodies HMFG-1 and HMFG-2 (Figure 2). Maximum binding of the second antibody was seen when HMFG-2 rather than HMFG-1 was used as the capture antibody. However as with the cell associated material, there appear to be more HMFG-1 reactive epitopes than HMFG-2 reactive epitopes on the bound antigen.

To see whether the secreted mucin contained the cytoplasmic tail, it was precipitated from the supernatant of ^{3}H-glucosamine labelled CDE2-1 cells with either the HMFG-1 monoclonal antibody or with a polyclonal antibody to the cytoplasmic tail, CT1. Precipitates were then run on a polyacrylamide gel, blotted and exposed to film. Cell lysates of CDE2-1 and MCF-7 cells were also immunoprecipitated and blotted in the same way, to provide a positive control for the CT1

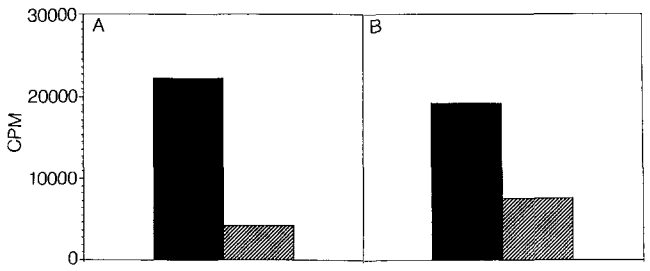

FIGURE 2
Radioimmunoassay of supernatants collected from CDE 2-1 (A) and CDE 2-3 cells (B). Supernatants were assayed using HMFG-2 as a capture antibody and either radiolabelled HMFG-1 (■) or HMFG-2 (▨) to detect bound antigen.

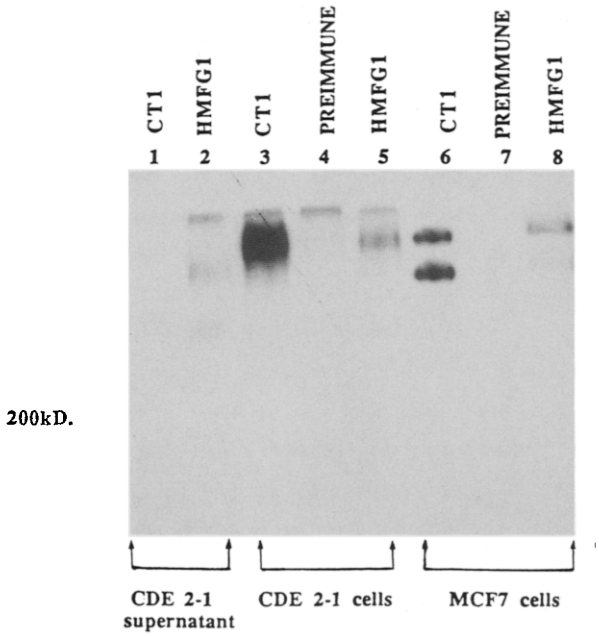

FIGURE 3
Immunoprecipitation of cell lines and supernatants. The antibodies used, HMFG-1, CT1 or preimmune rabbit serum (preimmune) are indicated on the top of the diagram, and the cell extract or supernatant at the bottom.

antibody. Figure 3 shows that the CT1 antibody does indeed precipitate PEM from cell lysates, as does the HMFG-1 antibody. However mucin was precipitated from the supernatant only by HMFG-1, and not by CT1. This experiment indicates that in this system, the secreted mucin is missing the cytoplasmic tail.

DISCUSSION

While partial sequences are now available for four human mucins (2-4, 23-25) the only mucin core protein for which the whole coding sequence is available is that coded for by the MUC1 gene. In this case, the sequence predicts a transmembrane protein, and the presence of an intracellular domain has been confirmed by the fact that positive staining with the CT1 antibody to the cytoplasmic tail is seen in fixed but not live cells (11). Although the sequences of the lung and intestinal mucin genes resemble MUC1 in having a domain made of tandem repeats (of different sequence and length for each mucin) the way in which they are attached to the cell surface is not yet clear. Indeed it is quite possible that these more complex, gel-forming mucins, are not transmembrane proteins and are secreted from internal vesicles.

Many membrane proteins are found in both a membrane bound and soluble form. In general terms these two forms can be generated by one of two mechanisms (26). The first involves separate biosynthetic pathways which may involve alternative RNA splicing, or transcription from separate genes, while the second involves release of the extracellular domain by cleavage of the membrane anchor. In the case of transmembrane proteins, examples of both alternative splicing and hydrolytic cleavage have been reported. In some cases, as for the c-erbB2 gene product, both alternative splicing (27) and post-translational cleavage (28) have been described. As a first approach to investigating the way in which a soluble form of the MUC1 gene product is produced, we have used mouse cells transfected with the full-length human MUC1 cDNA to ask whether release of the mucin can occur without the device of alternative splicing. We find that mucin is in fact released from the transfectants into the supernatant, and that the released material does not contain the intracellular domain found in the cell associated mucin. This result suggests that the soluble form of the MUC1 gene product can be generated by post-translational hydrolytic cleavage. Whether the same mechanism is operative in human cells normally expressing the MUC1 gene, remains to be clarified.

REFERENCES

1. Taylor-Papadimitriou, J. and Gendler, S.J. (1988) In, Cancer Reviews (J. Hilgers, S. Zotter, Eds.) Vol. 11-12, pp. 11-24, Munksgaard, Copenhager, Denmark.
2. Gendler, S.J., Lancaster, C.A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E.-N. and Wilson, D. (1990) J. Biol. Chem. 265, 15286-15293.
3. Wreschner, D.H., Hareuveni, M., Tsarfaty, I., Smorodinsky, N., Zaretsky, J., Kotkes, P., Weiss, M., Lathe, R., Bion, A. and Keydar, I. (1990) Eur. J. Biochem. 189, 463-473.
4. Ligtenberg, M.J.L., Vos, H.L., Gennissen, A.M.C. and Hilkens, J. (1990) J. Biol. Chem. 265, 5573-5578.
5. Lam M.S., Barra, S.K., Qi, W.-W., Metzgar, R.S. and Hollingsworth, M.A. (1990) J. Biol. Chem. 265, 15824-15830.
6. Swallow, D.H., Gendler, S., Griffiths, B., Corney, G., Taylor-Papadimitriou, J. and Bramwell, H. (1987) Nature 328, 82-84.
7. Burchell, J., Durbin, H., and Taylor-Papadimitriou, J. (1984) Int. J. Cancer 34, 763-768.
8. Linsley, P.S., Ochs, V., Laska, S., Horn, D., Ring, D.B., Frankel, A.E., and Brown, J.P. (1986) Cancer Res. 46, 5444-5450.
9. Hilkens, J., Bonfrer, J. and Kroezenv, M.G. (1987) Int. J. Cancer 39, 431-435.
10. Williams, C.J., Wreschner, D.H., Tanaka, A., Tsarfaty, I., Keydar, I. and Dion, A.S. (1990) Biochem. Biophys. Res. Commun. 170, 1331-1338.
11. Pemberton, L., Taylor-Papadimitriou, J. and Gendler, S.J. (1992) Biochem. Biophys. Res. Commun. 185, 167-175.

12. Miller, F.R., Miller, B.E. and Heppner, G.H. (1983) Invasion and Metastases 3, 22-31.
13. Soule, H.D., Vazquez, J., Long, A., Albert, S. and Brennan, M. (1973) J. Natl. Cancer Inst. 51, 1409-1416.
14. Burchell, J.M., Durbin, H. and Taylor-Papadimitriou, J. (1983) J. Immunol. 131, 508-513.
15. Burchell, J.M., Gendler, S., Taylor-Papadimitriou, J., Girling, A., Lewis, A. and Millis, R. (1987) Cancer Res. 47, 5476-5482.
16. Burchell, J., Taylor-Papadimitriou, J., Boshell, M., Gendler, S. and Duhig, T. (1989) Int. J. Cancer 44, 691-696.
17. Andersson, S., Davis, D.L., Dahlback, H., Jornvall, H. and Russell, D.W. (1989) J. Biol. Chem. 264, 8222-8229.
18. pSVneo plasmid
19. Graham, F.L. and Vander Eb, A.J. (1973) Virology 52, 456-467.
20. Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
21. Laemmli, U.K. (1970) Nature 227, 680-685.
22. Girling, A., Bartkova, J., Burchell, J., Gendler, S., Gillett, C. and Taylor-Papadimitriou, J. (1989) Int. J. Cancer 43, 1072-1076.
23. Gum, J.R., Byrd, J., Hicks, J., Toribara, N., Lamport, D. and Kim, Y. (1989) J. Biol. Chem. 264, 6480-6487.
24. Gum, J.R., Hicks, J., Swallow, D. et al. (1990) Biochem. Biophys. Res. Commun. 171, 407-15.
25. Porchet, N., Van Cong, N., Dufosse, J., Audie, J.P., Guyonnet-Duperat, V., Gross, M.S., Denis, C., Degand, P., Bernheim, A. and Aubert, J.P. (1991) Biochem. Biophys. Res. Commun. 175, 414-422.
26. Ehlers, M.R.W. and Riordan, J.F. (1991) Biochem. 30, 10065-10073.
27. Maihle, N.J., Flickinger, T.W., Raines, M.A., Sanders, M.L. and Kung, H.-J. (1991) Proc. Natl. Acad. Sci. USA. 88, 1825-1829.
28. Zabrecky, J.R., Lam, T., McKenzie, S.J. and Carney, W. (1991) J. Biol. Chem. 266, 1716-1720.