Episialin, a Carcinoma-associated Mucin, Is Generated by a Polymorphic Gene Encoding Splice Variants with Alternative Amino Termi

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Episialin is a mucin-type glycoprotein present at the luminal side of most glandular epithelial cells. We have isolated cDNA clones encoding episialin and determined the structure of the gene. The gene encodes a transmembrane protein which consists of, for the greater part, tandem repeats of 20 amino acids. The number of these repeats varies between 40 and 90 among different alleles. The repeats and most of the remainder of the protein are very rich in potential O-linked glycosylation sites. Two different splice variants were found. Interestingly, the proteins encoded by these two variants differ in their signal sequences and in the extreme amino-terminal parts of the mature proteins, suggesting alternative processing of these two species.

Episialin, previously called MAM-6 by our group, is a mucin which is abundantly present at the apical surface of epithelial cells. An increase in episialin expression is often found in carcinoma cells, where the glycoprotein is detected both intracellularly and on the entire cell surface, including the membranes lining the adjacent cells (Hilkens et al., 1984). The mucin has an apparent molecular mass of over 400 kDa on SDS-polyacrylamide gel electrophoresis. It contains many sialic acid residues, which result in a high net negative charge of the molecule (Hilkens and Buys, 1988). In combination with its high level of expression, this negative charge of episialin could have a significant effect on cell-cell interactions of carcinoma cells.

Previously, episialin has been defined by five monoclonal antibodies directed against different epitopes on the antigen (Hilkens et al., 1984, 1985). These antibodies detect episialin molecules of either one or two size classes in all episialin-synthesizing cell lines (Hilkens et al., 1989). The size of these molecules varies significantly between different individuals or cell lines. It has been shown that this variation in size is caused by a genetic polymorphism, which is inherited in a mendelian fashion (Swallow et al., 1987; Hayes et al., 1988).

The biosynthesis of episialin in mammary tumor cell lines has been studied extensively (Hilkens and Buys, 1988; Linsley et al., 1988). In ZR 75-1 cells, the molecular weights of the protein backbones of the two allelic forms were estimated to be 160,000 and 310,000, respectively, which is much lower than those of the mature glycoproteins. We have shown that the difference in molecular weight between the mature molecules and their precursors is caused by extensive glycosylation (Hilkens and Buys, 1988). Since most of the carbohydrate side chains are attached to the protein backbone by O-glycosidic linkages to threonine or serine residues, episialin is classified as a mucin.

Many other investigators have obtained monoclonal antibodies directed against this mucin (e.g. Taylor-Papadimitriou et al., 1981 (HMFG-2); Ashall et al., 1982 (CA1); Kufe et al., 1984 (DF3); Cordell et al., 1985 (anti-EMA); DeKretser et al., 1985 (OM-1); Burchell et al., 1987 (SM3)). In a previous study, we have shown that these monoclonal antibodies react with the same precursor molecules, although the reaction pattern toward mature episialin molecules differs among the various cell lines tested (Hilkens et al., 1988). Using the monoclonal antibodies SM3 and DF3, part of the episialin cDNA was cloned and was shown to contain repeats of 60 bp (Gendler et al., 1987, 1988; Siddiqui et al., 1988).

Mucins are glycoproteins that are difficult to study at the protein level due to their large size and their high number of glycosidic side chains. Therefore, molecular cloning of mucin genes is required to elucidate the complex structure and the biological function of these glycoproteins. However, no complete sequence for any mucin has been reported thus far. Here we present the full-length cDNA sequence and genomic organization of the episialin gene. Two splice variants are described here that differ from each other in their signal sequence and the region encoding the mature amino terminus, suggesting different processing of these two gene products.

MATERIALS AND METHODS

Cell Lines—For the isolation of RNA and DNA and for the immunoprecipitation of episialin, the following human breast carcinoma cell lines were used. ZR-75-1 cells were a gift from Dr. G. Malinckoff (Centocor Inc., Malvern, PA). T47D and MDA MB 157 cells were obtained from the American Type Culture Collection. BT-20 cells were kindly donated by Dr. J. Taylor-Papadimitriou (Imperial Cancer Research Fund, London). MCF-7 cells were obtained from Dr. F. Prop (University of Amsterdam). The bronchial alveolar carcinoma cell line A549 and the colon carcinoma cell line HT29 were obtained from the American Type Culture Collection. CEM cells were derived from a T cell leukemia and were donated by Dr. J. de Vries (Netherlands Cancer Institute, Amsterdam). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Monoclonal Antibodies—Monoclonal antibody 115F5 was raised against human milk fat globule membranes (Hilkens et al., 1984); monoclonal antibodies 158H2 and 140C1 were generated against

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05298.  

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§ The abbreviations used are: SDS, sodium dodecyl sulfate; bp, base pair(s); kb, kilobase(s).  

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primary breast cancer membranes (Hilkens et al., 1985). Monoclonal antibody SM3 was raised against the deglycosylated Human milk fat globule mucin (Burchell et al., 1987) and was kindly donated by Dr. J. Taylor-Papadimitriou and Dr. S. Gendler (Imperial Cancer Research Fund, London).

Immunoprecipitation and Southern and Northern Blotting—Labeling of cells with [3H]glucosamine and immunoprecipitation of episialin mRNA were synthesized as described before (Hilkens et al., 1984). DNA was isolated, separated on agarose gels, and transferred to nitrocellulose according to standard procedures. Unless otherwise stated, RNA was isolated using the lithium chloride/urea technique. Poly(A)+ RNA was isolated by chromatography using oligo(dT)-cellulose, separated on formaldehyde gels, and transferred to nitrocellulose according to standard procedures. Washing of the blots was performed under high stringency conditions.

Screening of the λgt11 Library—A random-primed cDNA library of the mammary tumor cell line T47D in λgt11 was obtained from Prof. P. Chambon (Institut de Chimie Biologique, Strasbourg). The library was plated onto Escherichia coli Y1090 (Promega Biotec). Screening was performed using hybridoma culture supernatants containing the monoclonal antibodies SM3, 139H2, 140C1, and 115F5. Monoclonal antibody binding was visualized using an alkaline phosphatase conjugate according to the method recommended by Promega Biotec. The obtained cDNA was used in the subsequent cloning procedures (see below).

RNA Size Fractionation and cDNA Cloning—RNA was isolated from the human breast cancer cell line T47D using the guanidine isothiocyanate/CoCl2 technique (Davis et al., 1986), and poly(A)+ RNA was purified by chromatography using oligo(dT)-cellulose. Poly(A)+ RNA (250 μg) was fractionated on a sucrose gradient, which was prepared by freeze-thawing of a 20% sucrose solution containing 50 mM sodium acetate (pH 5.2), 10 mM NaCl, and 1 mM EDTA (pH 8.0). Gradients were centrifuged in an SW41 rotor at 30,000 rpm for 14 h. Thirty fractions were collected, of which the presence of episialin RNA was analyzed on a Northern blot using a partial cDNA clone as a probe. Fractions containing either the 4.1- or 6.8-kb episialin messenger were pooled separately.

cDNA was ligated according to the method of Gubler and Hoffman (1983). Either the oligonucleotide GTGTCGGGGGCCGAGGTTG (based on the sequence of the initially obtained cDNA clone) or oligo(dT) was used as a primer. The cDNA was ligated via EcoRI linkers into phage λgt10 (Stratagene), packaged in vivo using Gigapack Gold (Stratagene), and transferred to E. coli CES 200 (RecB+). A agarose gel fractionation of DNA and genomic cloning—DNA of the T47D cell line was digested with EcoRI and separated on an agarose gel; fragments of the correct size class, as had been determined by Southern blotting, were isolated by electrophoresis. After purification by phenol extractions, the DNA was ligated into EcoRI-digested and -dephosphorylated λEMBL4 arms (Stratagene), packaged in vitro using Gigapack Gold (Stratagene), and transferred to E. coli KW225 (RecA-, RecB+). 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Fig. 2. Immunoprecipitation and Northern and Southern blot analyses. A, fluorogram of a 4-10% SDS-polyacrylamide gradient gel showing episialin immunoprecipitated with monoclonal antibody 139H2. The lysates were prepared from carcinoma cell lines that were labeled for 4 h with [3H]glucosamine. B, autoradiogram of a Northern blot carrying RNA of various cell lines hybridized with the episialin repetitive cDNA probe. The two upper bands present in lane C are not consistently found and probably represent unspliced RNA precursors. C, autoradiogram of a Southern blot carrying DNA of different cell lines digested with AluI, hybridized with the probe recognizing the repeat region of the episialin gene. Lane A, MCF-7; lane B, ZR-75-1; lane C, T47D; lane D, MDA MB 157; lane E, BT-20; lane F, CAMA-1 (breast carcinoma cell lines); lane G, A549 (a lung carcinoma cell line); lane H, Lucy (an ovarium carcinoma cell line); lane G*, HT29 (a colon cell line); lane H*, CEM (a T cell lymphoma cell line).

Fig. 3. Restriction map of two episialin alleles. Restriction maps are shown of uncloned DNA of the small allele (top) and of the large allele (bottom) of T47D cells. The hatched boxes indicate the regions that contain the repeats.

stream of the repeat region. When the nonrepetitive part of one of these cDNAs was used as a probe on Northern blots, the same hybridization pattern was observed as was observed for the repeat probe (data not shown). cDNA clones containing the region 3' of the repeats were isolated from an oligo(dT)-primed cDNA library, which was screened with the repetitive probe and genomic PstI fragments isolated from the genomic EcoRI clone (see below). Five cDNA inserts were analyzed containing identical regions of 1311 bp downstream of the repeat region. The combined length of the sequences on either side of the repeat region is about 1770 bp, which comes close to the estimated length of the nonrepetitive region (1.8 kb; see above).

Genomic Organization of Episialin Coding Region—The genomic EcoRI fragment, on which the repeat region of the small allele of the episialin gene in T47D cells is located, was cloned. The restriction map of this clone completely corresponds to the genomic map (Fig. 3) that was constructed using uncloned T47D DNA.

The sequence of part of the cloned fragment was determined and compared with the cDNA sequence. The deduced organization of exon and intron sequences is schematically shown in Fig. 4A. The second exon contains the entire repeat region and is extremely long, whereas 3' of this exon, several exons are found that are separated from each other by small introns. The last exon (exon 7) is encoded by another genomic EcoRI fragment (data not shown).

cDNA Sequence—Two different splice variants (A and B) have been obtained (Fig. 4). The variants are composites since we did not obtain cDNA clones spanning the entire repeat region. The flanking clones reached into the 5' or 3' ends of the repeat region. The combined sequence data as well as the deduced amino acid sequence are shown in Fig. 5. In this sequence, 40 repeat units are indicated, which is the estimated
Splice Variants—As indicated in Fig. 5, both splice variants show a great resemblance. The only difference is the use of two alternative splice acceptor sites for exon 2 that are located 27 bp apart (Fig. 4B). Therefore, the length of the putative translation products differs by only 9 amino acids. However, the alternative splicing event affects the signal sequences of the gene products. According to the predictive method of Von Heyne (1986), the signal peptide of variant A is 22 amino acids long and is cleaved between the threonine and alanine residues present in the additional 9 amino acids (score 11.11). The cleavage site of variant B is located between the glycine and serine residues 23 amino acids downstream of the translational start codon (score 12.75), resulting in a different amino terminal of the mature glycoprotein.

To determine the relative amounts of these splice variants in T47D cells, we have hybridized 28 cDNA clones, which were shown to contain both exon 1 and the common region in exon 2, with an oligonucleotide probe recognizing the additional 27 nucleotides present in variant A. Four out of these 28 cDNA clones hybridized with this oligonucleotide probe. This indicates that in T47D cells variant A containing these additional 27 nucleotides is less abundant than variant B.

DISCUSSION

We have obtained cDNA clones that, together with the genomic clone containing all the repeats, span the complete coding region of the epithelial mucin episialin. Clones containing the 60-bp repeats have been isolated by other groups using monoclonal antibody SM3 (Gendler et al., 1987) or DF3 (Siddiqui et al., 1988), which are directed against the same epithelial mucin. The nucleotide sequence of the repeats of these cDNA clones (Gendler et al., 1988; Siddiqui et al., 1988) is identical to the sequence we have determined, although the sequence of Siddiqui et al. is published in the reverse orientation. We have established the coding strand of our cDNA by hybridization of a Northern blot with oligonucleotides deduced from either of the two strands of the repeats (data not shown). The same direction of transcription has been
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The sequence 3′ of the repeats in our clone is completely different from the one reported by Gendler et al. (1988). When we compare the latter with our genomic sequence, alternative splicing can be excluded as an explanation for this difference. The polymorphism is reported by Gendler et al. (1988). When on the basis of the absence of a splice donor site immediately 3′ of the repeats. Restriction fragments derived from various parts of the cDNA described here recognize the same pattern in this paper totally represents episialin RNA. 

It has been found by others (Karlsson et al., 1983; Swallow et al., 1987; Hayes et al., 1988) that the variation in length of the AluI fragments in other cell lines varies in length is located entirely between the AluI sites that are due to different numbers of repeats. As can be estimated from the length of the AluI fragments that hybridize with the repeat probe, the number of repeats varies between about 40 and 90 among the different cell lines. Because of the large amount of potential O-linked glycosylation sites in the repeats, it is likely that the repeats mainly serve as a carrier for carbohydrate side chains. Recently, partial cDNA clones of other mucins have been reported. Both the porcine submaxillary gland apomucin (Timpte et al., 1988) and the human intestinal mucus have been reported. 

Fig. 5. cDNA sequence of variants A and B. Shown is the combined nucleotide sequence of the different cDNA clones and their protein products. The first nucleotide of the translational start codon is designated as +1. The numbering of the nucleotide sequence 3′ of the repeat is adopted to that of the small allele of T47D containing 40 repeats. Boldface amino acids represent the putative amino termini of the mature proteins. The blocked amino acid sequence indicates the transmembrane region. The polyadenylation sequence is underlined twice. The arrowheads indicate exon boundaries.

reported by Gendler et al. (1988).

The occurrence of N-linked glycosylation of episialin had already been established in the structural analysis and biosynthesis studies of episialin.
fore, the alternative splicing event not only gives rise to an alternate signal sequence, but also generates another amino terminus of the mature glycoprotein. This region may be important for the routing of the protein through the cell, perhaps resulting in different glycosylation pathways. This could explain the different cellular localizations of episialin that are detected in carcinoma cells (Kufe et al., 1984; Hilkens et al., 1984) and the distinct subpopulations of episialin that are found in sequential precipitation studies (Hilkens et al., 1989).

A potential transmembrane sequence is observed in the carboxyl-terminal part of the protein backbone of both splice variants. The presence of a potential transmembrane region in episialin was not expected since episialin has been detected in the spent medium of episialin-producing cell lines and in the serum of breast cancer patients (Hays et al., 1986; Hilkens et al., 1986). Whether the glycoprotein is released from the cell surface by an extracellular protease activity or is excreted in a membrane bound form is not yet known.

The only 3 cysteine residues occurring in the translation product are present in the transmembrane region. These residues are probably used for the addition of fatty acids to the molecule as has been reported before for other transmembrane glycoproteins (for review, see Magee et al., 1980). This acylation may stabilize the binding of the mucin in the membrane. Since episialin does not contain cysteine residues in the extracellular domain, it cannot form oligomers via disulfide bonds. This confirms our previous finding that reducing conditions have no major influence on the mobility of the glycoproteins on SDS-polyacrylamide gels (Hilkens et al., 1989). This lack of formation of disulfide bonds is in contrast with the oligomerization of the gel-forming mucins that are present in secretions from specialized epithelial cells in many exocrine tissues, such as the salivary gland (for review, see Hilkens, 1988). The absence of disulfide bonds and its tissue distribution distinguish episialin from these mucins.

The availability of the complete cDNA of episialin allows us to perform transfection studies to investigate the biological function of this mucin and to determine whether the different signal sequences can affect its subcellular distribution and glycosylation.

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