Cell-associated Episialin Is a Complex Containing Two Proteins Derived from a Common Precursor

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The abbreviations used are: SDS, sodium dodecyl sulfate; LRP, lipoprotein receptor-related protein; ASGP, ascites sialoglycoprotein.

Mucins are large molecules containing many O-linked glycans. The structure of the epithelial sialomucin episialin has been studied extensively. The availability of monoclonal antibodies directed against the protein backbone of episialin has made it possible to clone its cDNA and to study its biosynthesis. The cDNA sequence predicts that episialin is a type I membrane molecule with a large extracellular domain, which varies in length between approximately 1000 and 2200 amino acids, and a cytoplasmic domain of 69 amino acids (Ligtenberg et al., 1989; Abe and Kufe, 1989).

The biosynthesis of episialin has been described by several groups (Hilkens and Buijs, 1988; Linsley et al., 1988; Abe and Kufe, 1989). Both Hilkens and Buijs (1988) and Linsley et al. (1988) have observed that the molecular mass of the first detectable precursor is reduced by 20 kDa within 4 min. Hilkens and Buijs (1988) have proposed that this shift is the result of a proteolytic cleavage. This putative cleavage should take place in the endoplasmic reticulum, since it occurs shortly after translation. The mobility on SDS-polyacrylamide gels of the subsequent intermediate is drastically reduced, because a large number of O-linked sugars is added to the molecule. During the last step of the processing, the mobility of the episialin molecules is slightly altered by the addition of sialic acids. However, as a result of the high molecular weight of the molecules, an additional proteolytic cleavage at this stage could not be excluded (Hilkens and Buijs, 1988).

The presumed proteolytic cleavage in the endoplasmic reticulum should remove 20 kDa from either the N or the C terminus of the molecule. Based on the length of the different domains predicted by the cDNA sequence, cleavage in the C-terminal part of the molecule is expected to separate the actual mucin-like domain containing the repetitive region from the transmembrane and the cytoplasmic domain. This would require an alternative mechanism for the membrane association of this mucin-like domain. On the other hand, this cleavage could explain why the mucin-like molecule is released from carcinoma cells and found in serum of patients with breast cancer, in which it is demonstrated to be an important marker to monitor breast cancer therapy.

This study, we have analyzed the early proteolytic cleavage step in detail by comparing the processing of episialin synthesized in vitro and in vivo. We demonstrate that the cleavage indeed occurs in the C-terminal part of the molecule and that the resulting cleavage products remain associated, which explains the membrane anchorage of the mucin-like domain.

MATERIALS AND METHODS

cDNA Constructs—The generation of full-length cDNAs encoding episialin has been described previously (Ligtenberg et al., 1992). cDNA constructs containing a single repeat were generated from these full-length cDNA constructs of the A and B variant by exchanging a BsmI-EcoNI fragment of the cDNA constructs with a BsmI-
**Epitasil Complex Formation**

EcoRI fragment of a genomic clone that had lost all but one repeat during replication in a Rec+ bacterial strain. This recombination event had not affected the reading frame downstream of the repeat region.

The coding domains of these cDNAs were isolated by digestion with BamHI (located in the polylinker just 5′ of the cDNA and 18 nucleotides upstream of the polyadenylation signal) and inserted into the BamHI site of pGEM3Z (Promega Corp.). Plasmids containing the insert in both orientations were isolated. RNA could thus be made using either SP6 or T7 polymerase.

In *Vitro Transcription and Translation*—In *vitro* transcription and translation reactions were performed using SP6 or T7 polymerase and rabbit reticulocyte lysates according to the instructions of the manufacturer (Promega Corp.). When indicated, *in vitro* translation was stopped by addition of cycloheximide (100 μM). *In vitro* translation products were analyzed on standard SDS-polyacrylamide (Laemmli, 1970) or ZR-75-1 gels, as indicated in the figure legends.

**Generation of Polyclonal Antiserum and Immunoprecipitation**—The synthetic oligopeptide THGRYVPPSSTDRSPYE, which represents a region in the cytoplasmic domain, was coupled to keyhole limpet hemocyanin and used to immunize a rabbit. Antibodies directed against the C-terminal domain were purified from the polyclonal antiserum on an affinity column carrying a fusion protein consisting of the major part of β-galactosidase and part of the cytoplasmic domain of epitasil.

Pulse chase experiments and immunoprecipitations were performed essentially as described previously (Hilkens and Buijs, 1988). Cells were labeled with [35S]methionine and cytosolic lysates were isolated as described previously (Hilkens et al., 1986). Either monoclonal antibody 115D8 (Hilkens et al., 1984) or the purified polyclonal antibodies directed against part of the cytoplasmic domain were used as catcher. In both cases, monoclonal antibody 115D8 labeled with [35S]methionine was used as a tracer. Cell lysates were prepared in PBS containing 0.5% Nonidet P-40. Just before the assay, Triton X-100, sodium deoxycholate, and sodium dodecyl sulfate were added to the lysates to a final concentration of 1, 0.5, and 0.1%, respectively. Samples were added in triplicate to the wells either directly or after incubation at 100 °C for 10 min, followed by rapid cooling on ice.

**RESULTS**

*Epitasil Is Proteolytically Cleaved in Vitro*—To analyze the early proteolytic cleavage, *in vitro* synthesized RNA encoded by epitasil cDNA containing only one repeat was translated for 60 min in rabbit reticulocyte lysates. After labeling with [35S]methionine, the major translation product had a length of about 41 kDa. However, after labeling with [35S]cysteine, these products were not detected (results not shown). The full-length *in vitro* translation product was expected to be labeled with both methionine and cysteine, since the open reading frame contains 4 widely spread methionine residues (1 in the signal peptide, 2 in the remainder of the extracellular domain, and 1 in the cytoplasmic domain) and 3 cysteine residues that are all present in the transmembrane domain. The absence of a detectable product after labeling with [35S]cysteine suggested therefore, that the translation product did not contain the transmembrane domain. This might be caused either by removal of the C-terminal part of epitasil, including this domain after a full-length translation product has been synthesized, or by a strong translational stop signal in the mRNA, which can not be overcome by the translation machinery. To investigate whether the observed translation products were formed after a proteolytic cleavage, the translation was arrested after 22 min using cycloheximide, whereas the incubation of the translation mixture was continued. At various time points, samples of the *in vitro* translation reaction were taken and analyzed on a tricine-SDS-polyacrylamide gel (Fig. 1). After only 14 min of translation in both

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**Fig. 1. Cleavage of *in vitro* translated epitasil.** Autoradiogram of *in vitro* translated epitasil encoded by splice variant B separated on a tricine-SDS-polyacrylamide gel (10% spacer and 16.5% separating gel). The *in vitro* translation was performed in the presence of [35S]methionine (lanes 1–6) or [35S]cysteine (lanes 7–12). After 22 min, cycloheximide (100 μM) was added, and samples were taken at different time points (14, 30, 45, 90, 150, and 350 min, as indicated in the figure). Lanes 13 and 14 demonstrate two separate *in vitro* translation reactions, to which no cycloheximide was added, performed in the presence of [35S]cysteine for 70 min. In the reaction presented in lane 13, RNA derived from cDNA truncated at the Puul site was added; in lane 14, RNA derived from full-length cDNA was added. Only lanes 12, F, the full-length translation products; N and C, the N- and C-terminal cleavage products, respectively. (The band of about 30 kDa has been shown to represent a protein that is encoded by an open reading frame of 245 amino acids that starts downstream of the *in vivo* start codon in an alternative reading frame.)
the methionine- and cysteine-labeled samples, a major product with a length of about 68 kDa was detected (lanes 1 and 7). After inhibition of the translation and continued incubation, this band disappeared, and in the methionine-labeled samples, a band of about 41 kDa, which we had detected before, and a doublet of bands of about 20 kDa were observed (lanes 2–6), whereas in the cysteine-labeled samples only the doublet could be detected (lanes 8–12). This indicates that the 41-kDa translation product is indeed generated as a result of a proteolytic cleavage step, which removes the transmembrane region containing the cysteine residues and the cytoplasmic domain. The doublet of bands of 20 kDa, which should contain about a third of the methionine residues present in the larger N-terminal cleavage product, is relatively poorly visualized. This is probably the result of further degradation of the cleavage products, since a general loss of cysteine labeled products is observed on short exposures of the autoradiogram. Moreover, distortion of the electropherogram due to the presence of large amounts of unlabeled globin cannot be excluded.

To confirm that the doublet of bands of about 20 kDa represents the C-terminal cleavage product, RNA that was truncated at the PvuI site, which is located within the region encoding the cytoplasmic domain, was translated in the presence of [35S]cysteine for 70 min. The resulting translation products were compared with those of a similar reaction in which nontruncated RNA was used (compare lanes 13 and 14). Both the noncleaved precursor and the C-terminal cleavage product derived from the truncated RNA were expected to be about 3 kDa smaller than those derived from the full-length RNA. This shift in molecular mass was indeed observed for the noncleaved precursor. Upon truncation, the doublet of bands of about 20 kDa was absent, which supports the assumption that this doublet represents the C-terminal cleavage product. No alternative C-terminal fragment derived from the truncated molecule was observed, probably because it comigrates with the large amount of globin present in reticulocyte lysates. The appearance of the C-terminal cleavage product as a doublet might be the result of the use of two closely spaced cleavage sites or of two consecutive cleavage events.

**Determination of the Site of Proteolytic Cleavage in Vitro**—To localize the amino acid sequence that is the target of the observed proteolytic cleavage, RNA was synthesized from the cDNA constructs of both variants A and B, which were truncated at different restriction sites, as indicated in Fig. 2A. Translation reactions were performed for 35 min to obtain a mixture of precursor and proteolytically cleaved translation products (Fig. 2B). All products were compared with the full-length translation products of either the A or the B variant (about 59 and 58 kDa, respectively) and their proteolytically cleaved products (about 42 and 41 kDa, respectively) (lanes 6 and 7). The translation products encoded by the cDNAs truncated at or upstream of the SspI sites are smaller than these proteolytic cleavage products (lanes 1–3 and 10–13), whereas the precursors encoded by the cDNAs truncated at the 3′ KpnI and the PvuI sites are larger than these cleavage products (lanes 4, 5, 8, and 9). This pinpoints the cleavage site of both variant A and B to a stretch of 18 amino acids encoded by the region of the cDNA located between the SspI and the 3′ KpnI site. This area is located in the extracellular domain, 71 to 53 amino acids upstream of the transmembrane domain. The length of the resulting C-terminal cleavage product is approximately 160 amino acids, which is in good agreement with its estimated molecular mass of about 20 kDa on the tricine-SDS-polyacrylamide gel (Fig. 1). Since the proteolytic cleavage site maps within the same region for both splice variants, in all further experiments only splice variant A was used.

The issue of cotranslational membrane insertion as a factor that could affect the proteolytic cleavage(s) observed was addressed by performing similar in vitro translation experiments but now in a system supplemented with microsomal membranes. The translation products were analyzed after N-glycanase treatment to remove the N-linked glycans. The size of the larger proteolytic cleavage product was again slightly smaller than that observed for the translation product derived from the cDNA truncated at the 3′ KpnI site (results not shown). This suggests that the cleavage occurs at the same site independent of the presence of microsomal membranes. The structural elements recognized by the proteolytic machinery are therefore affected neither by membrane insertion nor by N-linked glycosylation.

**Proteolytic Cleavage of Episialin in Vivo**—To check whether the proteolytic cleavage observed in vitro is indeed similar to the cleavage that has been reported to occur in the endoplasmic reticulum, a polyclonal antiserum was raised against a synthetic peptide with a sequence identical with a stretch of 17 amino acids located in the cytoplasmic tail of episialin. If a similar cleavage would occur in vivo, the cytoplasmic domain would be separated from the extracellular domain containing the repeat region, and it would thus be expected that the latter domain would not be immunoprecipitated by the polyclonal antiserum. To test this hypothesis, the human breast carcinoma cell line T47D was labeled with [3H]threonine for 20 min followed by chase periods of 0, 20, 40, 60, 90 or 180 min. Cell lysates were immunoprecipitated with monoclonal antibody 139H2 directed against the repeat region and with the polyclonal antiserum directed against part of the...
cytoplasmic domain. Both the monoclonal and polyclonal antiserum immunoprecipitated the precursor and mature forms of both the large and small alleles (Fig. 3), suggesting that the observed decrease in molecular mass of about 20 kDa in the endoplasmic reticulum was not the result of a proteolytic cleavage at the site we had determined in vitro. However, an alternative explanation would be that the two proteolytic cleavage products form a complex that remains intact during the immunoprecipitation but dissociates during the preparation of the samples for SDS-polyacrylamide gel electrophoresis. The C-terminal cleavage product might easily have remained undetected because of its small size and its low content of threonine residues. To investigate whether such a complex indeed existed, the human breast carcinoma cell line ZR-75-1 was labeled with [3H]glucosamine, after which the cells were lysed in PBS containing 0.5% Nonidet P-40. To disrupt putative protein complexes prior to immunoprecipitation, the cell lysate was treated with either 6 M urea, 5% β-mercaptoethanol, or 1% SDS, as described under “Materials and Methods.” Immunoprecipitations were performed using either a monoclonal antibody directed against the repeats (139H2) or the polyclonal antiserum that recognizes the cytoplasmic domain (Fig. 4A). Comparable amounts of episialin could be immunoprecipitated with 139H2, irrespective of the treatment of the cell lysate. Using the anti-C-terminal antibody, episialin was immunoprecipitated after treatment with β-mercaptoethanol and urea, but not after treatment with SDS. Apparently, a complex is formed between the extracellular domain and the remainder of the molecule containing the cytoplasmic domain, which is dissociated in the presence of 1% SDS but not in the presence of 6 M urea or β-mercaptoethanol. (The latter treatment was not expected to dissociate the complex, since the only 3 cysteine residues are located closely together in the transmembrane domain, making disulfide bonds between two possible cleavage products highly unlikely.) Unfortunately, in contrast to the in vitro translation experiments, it turned out to be difficult to efficiently label episialin in vivo with a radioactive amino acid, which should enable us to visualize the putative C-terminal cleavage product on a polyacrylamide gel. However, an independent confirmation for the presence of a complex in vivo is presented below.

To obtain additional evidence for the formation of a complex, we examined whether a similar complex could be formed in vitro. Therefore, we tested whether the N-terminal proteolytic cleavage product of the in vitro translated material could be immunoprecipitated by the anti-C-terminal antiserum. As demonstrated in Fig. 4B, not only the noncleaved translation product and the doublet of bands representing the C terminus but also the N-terminal cleavage product could be immunoprecipitated with the polyclonal antiserum directed against the cytoplasmic domain (lane 3). This was neither caused by cleavage of the precursor after the immunoprecipitation, which was not observed, nor by association of the N-terminal cleavage product with the noncleaved precursor, since the N-terminal cleavage product was also immunoprecipitated from a sample that was almost completely cleaved at the start of the immunoprecipitation (lanes 5 and 7). To exclude that the N-terminal cleavage product was immunoprecipitated as a
result of cross-reactivity of the antiserum, an *in vitro* translation product that is encoded by a cDNA truncated at the *Ball* site and thus contains the entire extracellular domain but lacks the cytoplasmic domain (see Fig. 2A), was made. As expected, this translation product could not be immunoprecipitated using the polyclonal antiserum directed against the cytoplasmic tail (lanes 4 and 8). These results prove that the N-terminal cleavage product is immunoprecipitated by the antibody recognizing the cytoplasmic domain as a result of the formation of a complex between both cleavage products. Since this stable association is found both *in vitro* and *in vivo*, and since the interaction is expected to be dependent on the structure of the proteins near the cleavage site, it is likely that episialin is cleaved similarly in both systems.

The Proteolytic Cleavage Site Can Be Deleted from Episialin—To support our assumption that a similar cleavage occurs *in vivo* and *in vitro*, a region of 28 amino acids comprising the cleavage site used *in vitro* was deleted in both the constructs used for the *in vitro* translations containing only one repeat and in episialin cDNA containing the entire repeat region, which was used for transfection of HBL-100 cells (see Fig. 5A). In contrast to the wild-type molecule, the *in vitro* translation product derived from the deletion mutant was not converted to a discrete smaller product, even after prolonged incubation, as was to be expected following the removal of the specific cleavage site (compare Fig. 5B, lanes 1, 3, and 5 and lanes 2, 4, and 6, respectively). The *in vitro* translated mutant molecule seems to be instable, since its amount is markedly reduced during the incubation period.

For the *in vivo* experiments, both wild-type and mutant episialin cDNAs containing the entire repeat region were cloned downstream of the cytomegalovirus immediate-early promoter in the vector pCMVIE-AK1-DHFR (Whang et al., 1987), of which the region containing the dihydrofolate reductase gene had been removed. Both constructs were transfected into the SV40-transformed human mammary epithelial cell line HBL-100. G418-resistant clones were analyzed for the expression of episialin by membrane immunofluorescence, and the presence of the deletion was confirmed by Southern blotting (results not shown). The biosynthesis of both wild-type and mutant episialin was studied in these transfectants. Cells were labeled with [3H]threonine for 5 min and lysed directly or chased for an additional 15 min (Fig. 5C). The molecular mass of the wild-type molecule decreased with approximately 20 kDa during the 15-min chase period (compare lane 1 with lane 2). During this chase period, no shift in the molecular mass was observed for the mutant molecule (compare lane 3 with lane 4). Both after the 0- and 15-min chase periods, a band of a relatively low intensity was observed with a molecular weight similar to the proteolytically cleaved wild-type molecule. However, this band varied in intensity among different experiments, and in contrast to what was observed for the wild-type molecule, the intensity of this band did not increase after the 15-min chase period. Therefore, it probably is the result of proteolytic degradation during cell lysis or immunoprecipitation, as has been observed before (Hilkens and Buijs, 1988). This indicates that in contrast to wild-type episialin, the mutant molecules are not proteolytically cleaved in the endoplasmic reticulum. The similar behavior of the mutant molecules in *vivo* and *in vitro* implies that the proteolytic cleavage site *in vivo* is located at the same site or in close vicinity to the site used *in vitro*.

Evidence for the Association of the Cleavage Products in Vivo—To confirm that the wild-type molecules are present as complexes, whereas the entire extracellular domain is intact in the mutant molecules and is thus covalently linked to the cytoplasmic tail, a sandwich radioimmunoassay was performed. Either monoclonal antibody 115D8, directed against an epitope in the repetitive region, or the polyclonal antiserum recognizing the cytoplasmic domain were used as a catcher, and 125I-labeled 115D8 was used as a tracer. Cell lysates of five different wild-type and mutant transfectants and of the parental cell line HBL-100 were tested either before or after boiling of the samples to dissociate existing complexes. The results of the assay are shown in Table I. Boiling of the antigen does not affect the epitope recognized by monoclonal antibody 115D8, since the binding of the antibody was similar before and after boiling of the antigen. The same holds true for the polyclonal antiserum, since the amount of mutant episialin detected before and after boiling of the samples is essentially the same. However, after boiling of the cell lysates containing wild-type episialin, hardly any molecules could be detected by the tracer 115D8 when the polyclonal antiserum was used as a catcher. We conclude that upon heat treatment the repeat region is detached from the cytoplasmic domain in wild-type episialin, whereas these regions remain associated in the mutant molecules that were shown to be resistant to the proteolytic cleavage. This confirms that the N- and C-terminal cleavage products of wild-type episialin, which are
Episialin Complex Formation

### DISCUSSION

The results of the in *vitro* translation assays and the analysis of the mutant episialin molecules demonstrate that episialin is synthesized as a transmembrane molecule, which is cleaved in the endoplasmic reticulum to generate two subunits that remain associated through what appear to be noncovalent interactions. The N-terminal subunit comprises the major part of the extracellular domain, including the repetitive region. The C-terminal domain is much smaller and is often occurs directly downstream of two consecutive basic amino acids (Sossin et al., 1989; Barr, 1991) or after the second glycine in the sequence Gly-Gly-X, where X is often an amino acid residue with a hydrophobic side chain (López-Otin et al., 1989). No such sequences are present in the region to which the cleavage has been mapped in *vitro*, but the latter motif is found close to this region upstream of the position, which corresponds to the Spl site in the cDNA. This putative recognition site has been deleted in the mutant that has proven to be resistant to the cleavage in *vivo*. Therefore, we can not formally exclude that this site is used in *vivo*, although this would imply that the *in vitro* and *in vivo* cleavage sites are not identical. In the region to which the cleavage has been mapped in both systems, two Phe-Arg dipeptides are present, which are putative substrates for a family of serine proteases, the kallikreins (Fiedler et al., 1987). Cleavage at these sites could explain the presence of a doublet of bands representing the C-terminal cleavage product in the *in vitro* translation experiments. Amino acid sequencing of the N terminus of the C-terminal subunit should elucidate the exact cleavage site(s) and could give us a key to the identification of the protease(s) responsible for the cleavage(s).

The stable association of both cleavage products implies that an additional step is needed to release episialin from the cell surface, as is observed both in *vitro* and *in vivo*. This can either be the result of a second proteolytic cleavage of cell surface episialin or of the dissociation of the complex. In the latter case, transfectants expressing the mutant that is not cleaved would be expected to release hardly any episialin.

However, these transfectants were found to release at least as much episialin as those expressing the wild-type form (results not shown). This might reflect the reduced stability of the mutant molecule, which we have observed in the *in vitro* translation system and which is probably caused by an alteration of the three-dimensional structure of the mutant. It is also conceivable that a second cleavage is involved in the release of the molecule, since we have evidence that cell surface-associated episialin can be internalized and cycled back to the cell surface. During this recycling process, the molecule is likely to pass compartments that are rich in carbohydrate side chains. The remainder of the extracellular
domain is expected to be less sensitive to proteases, since its protein backbone is protected by closely packed glycans. Upon arrival at the cell surface, this part of the molecule might be released, because it is no longer anchored to the membrane.

For many other molecules, it is known that two subunits remain associated upon proteolytic cleavage of a common precursor. However, most of these molecules, such as some of the α-chains of the integrins (for review, see Hynes (1987)), several proteases and blood clotting factors, and the receptors for insulin (Deutsch et al., 1983; Hedo et al., 1983; Ronnett et al., 1984) and insulin-like growth factor-I (Jacobs et al., 1983), are linked by disulfide bonds. Like episialin, low density lipoprotein receptor-related protein (LRP) (Herz et al., 1990) and the ascites sialoglycoprotein (ASGP) complex (Sheng et al., 1990) consist of two subunits that are derived from a common precursor and that are associated through noncovalent interactions. The cleavage of LRP was shown to occur after the molecule had reached the Golgi complex. Sequencing of the N terminus of its C-terminal subunit has established that the cleavage takes place immediately downstream of 2 consecutive arginine residues. This suggests that the proteases involved in the cleavage of LRP and episialin are different. Ascites sialoglycoprotein-1, the N-terminal part of the ASGP-precur sor, has many biochemical properties in common with episialin. The molecule, which was characterized in 13762 rat ascites mammary adenocarcinoma cells, has a high molecular mass (about 600 kDa) as a result of extensive O-linked glycosylation and sialylation. The molecule forms a complex with ASGP-2, a membrane-spanning molecule of 120 kDa carrying many N-linked sugars. As has been established for episialin (Hilkens and Buijs, 1988), both the common precursor of ASGP-1 and -2 and the earliest detectable form of ASGP-2 carry N-linked sugars of the high mannose type. This suggests that the ASGP precursor is cleaved before it reaches the medial Golgi, where conversion of high mannose to complex oligosaccharides might occur. Why LRP, ASGP, and episialin are proteolytically processed is not known. Both ASGP-1 and episialin are eventually released from the cell surface, which might be caused by dissociation of the complex. Identification of the function of the released episialin molecules might reveal the logic behind the formation of this unusual complex.

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