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Carcinoembryonic antigen is anchored to membranes by covalent attachment to a glycosylphosphatidylinositol moiety: Identification of the ethanolamine linkage site

(posttranslational modification/ethanolamine linkage/fluorescent flow cytometry/microsequence analysis/fast atom bombardment mass spectrometry)

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ABSTRACT **The COOH-terminal amino acid of carcinoembryonic antigen (CEA) is shown to covalently link with ethanolamine, evidence consistent with the anchorage of CEA to the plasma membrane through a phosphatidylinositol-glycan tail. Purified CEA was digested with trypsin, and the resulting peptides were isolated by reverse-phase HPLC. Tryptic hexapeptide T12, terminating atypically with alanine, corresponded in sequence (Ser-Ile-Thr-Val-Ser-Ala) with the last six residues (637–642) of the third repeating domain in the mature CEA protein. Mass determination of the hexapeptide by fast atom bombardment mass spectrometry suggested the presence of an additional ethanolamine moiety. This finding and the absence of the subsequent 26 hydrophobic residues predicted by cDNA sequence is evidence that hexapeptide T12 is the COOH-terminal peptide of mature CEA. A synthetic peptide identical to hexapeptide T12 was prepared, and ethanolamine was coupled to its COOH-terminal alanine; chromatographic properties of this synthetic ethanolamine-coupled peptide and peptide T12 were the same. B/E-linked-scan mass spectral analysis of the ethanolamine-coupled synthetic peptide and peptide T12 revealed a fragment ion series consistent with the presence of a COOH-terminal ethanolamine. Release of membrane-bound CEA from the CEA-expressing cell line LS 174T was shown by indirect immunofluorescence and flow cytometry after treatment with phosphatidylinositol-specific phospholipase C. We conclude that CEA is processed posttranslationally to remove the hydrophobic COOH-terminal residues (643–668) with subsequent addition of an ethanolamine-glycosylphosphatidylinositol moiety and that treatment of a colonic cell line with phosphatidylinositol-specific phospholipase C releases membrane-bound CEA.**

Carcinoembryonic antigen (CEA) is one of the most thoroughly characterized human tumor-associated antigens for chemical, biochemical, tissue distribution, and clinical aspects (for review, see ref. 1). The complete amino acid sequence for CEA, determined by cDNA (3) sequencing, shows that CEA has a 34-amino acid leader sequence, followed by a 107-amino acid NH_2-terminal domain, three highly homologous repeating domains of 178 residues, and a 26-amino acid hydrophobic COOH-terminal domain. The three repeating domains share extensive sequence homology with the immunoglobulin gene superfamily, suggesting an evolutionary relationship (2). The hydrophobic COOH-terminal segment has been suggested as the membrane-anchoring domain (3). CEA contains 28 asparagine-linked glycosylation sites and is ≈50% carbohydrate by weight. Several antigens have been found that are related structurally and immunologically to CEA, which include the nonspecific crossreacting antigens NCA 55, NCA 95, and NCA 2 (4–9); biliary glycoprotein I (10); a 128-kDa antigen (11); a 100-kDa meconium antigen (12); and several other antigens found in feces (13).

Protein sequencing confirmed the cDNA-derived sequence predicted (3) for the NH_2 terminus and three immunoglobulin-like repeating domains and identified the 28 asparagine-linked glycosylation sites (2). No peptides from chymotryptic or tryptic digestions were isolated that corresponded to the predicted sequence of the COOH-terminal domain. Thus, either the mature protein lacked this proposed membrane-anchoring domain, requiring the protein to be anchored by another mechanism than direct peptide insertion, or the predicted domain was lost during purification.

Recently, a mechanism for attachment of proteins to membranes has been described that involves a complex phosphatidylinositol-glycan (so-called PI-G) tail covalently linked through ethanolamine to the COOH-terminal residue of the mature protein (for review, see ref. 14). Currently, >20 proteins are thought to be so anchored. Evidence for this type of attachment includes release of the membrane-bound protein with phosphatidylinositol-specific phospholipase C (PtdIns-PLC), chemical identification of some components in the complex, and observation that a relatively short hydrophobic peptide is missing from the COOH-terminus of the mature protein (14). Although the exact processing mechanism(s) is unknown, a "transamidase" enzyme is believed to remove a COOH-terminal segment from the protein and attach a phosphatidylinositol-glycan. Recent evidence (15, 16) suggests that the COOH-terminal domain acts as a "signal peptide" for processing by the transamidase, although no consensus-sequence signal is apparent. Existence of such a class of proteins prompted us to examine whether CEA was attached similarly to the lipid membrane.

From tryptic maps, we isolated a hexapeptide (T12), which agreed with the cDNA-predicted sequence (3) for the terminal six residues (637–642) of the third immunoglobulin-like repeating domain. This region is adjacent to the predicted COOH-terminal domain of CEA. Analysis of T12 by fast atom bombardment MS (FABMS) suggested the presence of an additional ethanolamine moiety. Presence of ethanolamine was confirmed by comparing chromatographic and mass spectral properties of hexapeptide T12 and an ethanolamine-coupled synthetic peptide. To demonstrate that CEA was attached to membranes with a phosphatidylinositol-con-

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Abbreviations: CEA, carcinoembryonic antigen; FABMS, fast atom bombardment mass spectrometry; N-CAM, neural cell adhesion molecule; PtdIns-PLC, phosphatidylinositol phospholipase C.

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taining anchor, we cleaved CEA from the membranes of colonic carcinoma cells using PtdIns-PLC.

MATERIALS AND METHODS

Preparation of CEA Tryptic Maps. CEA was isolated from liver metastases of colon tumors and purified as described (17). Deglycosylation of the isolated protein was accomplished with anhydrous hydrofluoric acid (18) followed by dialysis against 10% (vol/vol) aqueous pyridine. Deglycosylated CEA was reduced with dithiothreitol and carboxymethylated with iodoacetic acid (19). The carboxymethylated-deglycosylated CEA sample was dialyzed against 0.2 M ammonium bicarbonate buffer, pH 7.8, and digested with purified L-1-tosylamido-2-phenylethylchloromethyl-ketone-treated trypsin (Worthington) at a protein/enzyme ratio of 50:1 (wt/wt) for 18 hr at 37°C. The tryptic peptides were separated by reverse-phase HPLC with a Vydac C_{18} (4.6 × 250 mm) column using a linear gradient of acetonitrile in 0.1% (vol/vol) trifluoroacetic acid.

Microsequence Analysis. Peptides were spotted on a poly(vinylidene difluoride) membrane (Millipore) and subjected to automated Edman degradation on a gas-phase microsequencer built at the City of Hope (20). The phenylthiohydantoin amino acid derivatives were identified by on-line reverse-phase HPLC. Amino acid compositions were determined using a Beckman 6300 amino acid analyzer after hydrolysis in 6 M HCl containing 0.2% 2-mercaptoethanol at 110°C for 48 hr.

FABMS. Samples were concentrated to dryness in poly(propylene) microcentrifuge tubes using a vacuum centrifuge, redissolved in 2 μl of 5% (vol/vol) aqueous acetic acid, and added to 2 μl of glycerol on a 1.5 × 6-mm stainless-steel sample stage. Positive-ion FAB mass spectra were obtained using a JEOL HX-100HF high-resolution, double-focusing, magnetic-sector mass spectrometer operating at 5-kV accelerating potential and a nominal resolution of 3000 or 5000. Sample ionization was accomplished using a 6-keV Xe atom beam. Data was collected with a JEOL DA5000 data system. For normal spectra, the electric sector of the mass spectrometer was set to transmit all ions at source potential (5 kV), and the magnetic field was scanned over a given mass range. For B/E-linked scans (21), the electric-sector voltage and magnetic-sector field strength were linked such that the ratio was constant.

Preparation of the Ethanolamine-Coupled Synthetic Peptide. The peptide (NH_2-Ser-Ile-Thr-Val-Ser-Ala) was synthetized by standard solid-phase methodologies (22) using Fmoc-Ala *p*-alkoxybenzyl polystyrene resin ester (23). The coupling reactions were accomplished in dichloromethane dimethylformamide using dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. The amino acids were amino-protected with the *N*-fluorenylmethoxycarbonyl group (Fmoc), with the side chains of serine and threonine protected with *t*-butyl groups. Coupling efficiencies of >99% were obtained as determined by a ninhydrin color test. The peptide was cleaved from the solid support using dichloromethane/trifluoroacetic acid, 3:7 (vol/vol), containing anisole (22) for 2 hr at 25°C. The resin was removed by filtration, the organic reagents were evaporated under vacuum, and the peptide was precipitated with ether. Purification of the peptide was accomplished by reverse-phase HPLC using a Vydac C_{18} (4.6 × 250 mm) column with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The purified peptide was lyophilized to dryness and redissolved in water (1 mg/ml) for coupling with ethanolamine. A 10-fold molar excess of redistilled ethanolamine was added to the peptide, and the mixture was titrated to a pH value of 4.7 with addition of 0.5 M HCl. A 1.5-fold molar excess (per peptide equivalent) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce) was added to the reaction mixture, and the pH was maintained at 4.7 with addition of 0.5 M HCl or ethanolamine for 1 hr (24). The reaction was stopped, and the product was purified by reverse-phase HPLC as described above.

Immunofluorescence Studies. The human colon adenocarcinoma cell line LS 174T (CL 188) and the Swiss mouse embryonic cell line NIH 3T3 (CRL 1658) were obtained from American Type Culture Collection. For treatment with PtdIns-PLC, 6×10^6 LS 174T and 1×10^6 NIH 3T3 cells were resuspended in Dulbecco's phosphate-buffered saline PBS (D-PBS) containing ovalbumin at 1 mg/ml (fraction V, Sigma), and 2 units of PtdIns-PLC from *Bacillus thuringiensis* (provided by S. Udenfriend) were added. One unit of enzyme is defined as that amount necessary to hydrolyze 8×10^{-4} μmol of phosphatidylinositol in 1 min at 37°C. Cells were incubated at 37°C for 1 hr and washed three times with cold D-PBS for analysis by fluorescence flow cytometry. Control LS 174T and NIH 3T3 cells were incubated in the same fashion but without addition of PtdIns-PLC. For analysis with fluorescence flow cytometry, half the population of treated and control cells were incubated with excess anti-CEA monoclonal antibody T84.1 (25, 26) for 1 hr at 4°C in D-PBS, washed three times with cold D-PBS, and incubated with excess fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ (Polyscience, Warrington, PA) for 1 hr at 4°C in D-PBS; the other half was incubated only with the fluorescein isothiocyanate-conjugated antibody. Cells were again washed three times, resuspended in cold D-PBS, and analyzed with a Becton Dickinson FACS IV flow cytometer.

RESULTS

Peptide Mapping. CEA was purified as described and deglycosylated with anhydrous HF to insure complete digestion with trypsin. Deglycosylation with HF hydrolyzes O-glycosyl bonds and leaves a single residue of N-linked *N*-acetylglucosamine at each glycosylation site. The modified asparagine residues can directly identify the glycosylation sites in CEA (2, 18).

The tryptic map for CEA is shown in Fig. 1. The peptides which eluted early in the gradient were generally well separated and could be sequenced without extensive rechromatographic steps. The later-eluting peptides were less well resolved, owing partially to their size and degree of hydrophobicity, and these peptides required extensive rechromatographic steps before sequence analysis (data not shown).

Trypic peptide T12 is indicated in Fig. 1. The peptide was rechromatographed to purity. Through direct protein sequencing and amino acid analysis, peptide T12 was found (Table 1) to correspond to residues 637–642 in CEA (3). This region of CEA is at the end of the third immunoglobulin-like

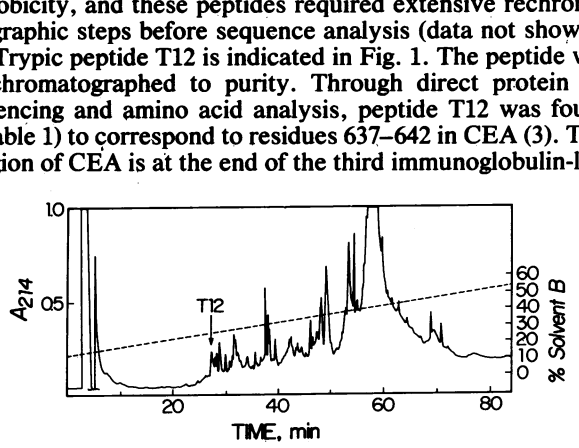

FIG. 1. Tryptic map of HF-treated CEA. A 3-nmol sample of carboxymethylated deglycosylated CEA was digested with trypsin at 37°C for 18 hr. The digest was applied to a Vydac C_{18} (4.6 × 250 mm) column, and the peptides were eluted with a linear gradient of 90% (vol/vol) buffer A/10% (vol/vol) buffer B to 40% (vol/vol) buffer A/60% (vol/vol) buffer B in 120 min. Buffer A is 0.1% trifluoroacetic acid in H_2O, and buffer B is 0.1% trifluoroacetic acid/9.9% (vol/vol) H_2O/90% acetonitrile. Solid line, A_{214}; dashed line, % buffer B.

Table 1. Sequence and composition analyses

Sequence analysis			Amino acid composition	
Cycle	Residue	pmol	Residue	pmol
1	Ser	19	Thr	164
2	Thr	55	Ser	397
3	Ile	80	Ala	203
4	Val	60	Val	200
5	Ser	2	Ile	184
6	Ala	4		

For amino acid-composition analysis, the sample (≈200 pmol) was hydrolyzed in 6 M HCl containing 0.2% 2-mercaptoethanol at 110°C for 48 hr. For sequence analysis, the sample (120 pmol) was spotted on a poly(vinylidine difluoride) membrane (1 × 10 mm) in a continuous-flow reactor and subjected to automated Edman degradation with a microsequencer.

repeating domain and is adjacent to the predicted COOH-terminal domain. Mass spectral analysis (see below) of peptide T12, however, suggested the presence of an additional ethanolamine moiety.

A synthetic peptide with the same sequence as peptide T12 was prepared, and ethanolamine was coupled to the COOH-terminal alanine. Chromatographic properties of peptide T12, the synthetic peptide, and the ethanolamine-coupled synthetic peptide are compared in Fig. 2 *A* and *B*. Presence of ethanolamine at the COOH-terminus decreases retention time of the synthetic peptide on a C_{18} reverse-phase HPLC column (Fig. 2*A*). The elution profile of peptide T12 is shown in Fig. 2*B*. The major peak, marked T12, had an identical retention time as the ethanolamine-coupled synthetic peptide. Also shown in Fig. 2*B* are two minor peaks eluting after T12; one of these peaks appeared to have a retention time similar to the underivatized synthetic peptide. Microsequence and FAB-MS analyses of these peaks, however, failed to detect any peptide sequences or to identify any compounds in the mass range analyzed (200–4000) that could account for their presence.

FABMS Analysis of Peptide T12. FABMS analysis of a 100-pmol aliquot of T12 in a glycerol matrix gave an intense protonated molecular ion at mass-to-charge ratio (*m/z*) 620. This mass was 43 mass units higher than the mass predicted from the composition data of peptide T12. An increase of 43 mass units suggested that an additional ethanolamine moiety

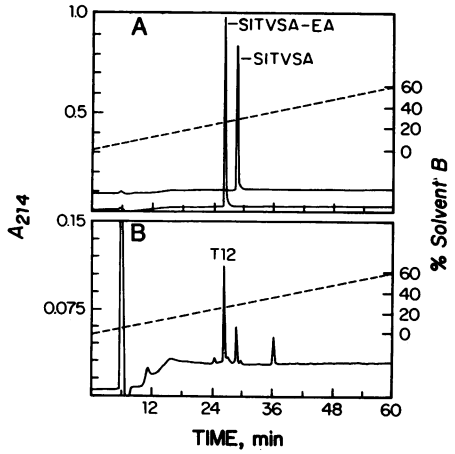

FIG. 2. Comparison of the chromatographic properties of a synthetic peptide (SITVSA), the same peptide coupled with ethanolamine (SITVSA-EA), and the natural tryptic peptide (T12) on a Vydac C_{18} (2.1 × 250 mm) column. Peptides were eluted with a linear gradient of 100% buffer A to 40% buffer A/60% buffer B in 60 min. Buffers are described in the legend for Fig. 1. Solid line, A_{214}; dashed line, % buffer B.

was linked to the COOH-terminal alanine through an amide bond. Glycerol matrix ions of known composition in the spectra permitted an exact mass measurement of peptide T12; the exact mass measured for T12 was 620.3595. This observed mass agreed well with the predicted mass (620.3619) for peptide T12 with ethanolamine coupled covalently to the COOH-terminal alanine.

At the sample levels analyzed, fragment ions in the FAB spectrum are normally obscured by ions from the matrix. Through a technique known as B/E-linked scan (21), however, a spectrum of the daughter ions generated from decomposition of a preselected parent ion can be obtained. A more complete description of the uses of B/E-linked scan mass spectrometry and of the possible ions derived from decomposition of peptides is provided elsewhere (27, 28).

Fig. 3 shows the results of the B/E-linked scan on 126 pmol of peptide T12 (*m/z* 620); the insert is provided to aid in identification of the NH_2-terminal and COOH-terminal fragment ion series. Ions corresponding to these series are labeled in the figure; of particular interest to this analysis is the ion at *m/z* 559. This ion arose from the loss of ethanolamine from the peptidyl portion of T12. As illustrated, this type of analysis provides enough information to define the entire peptide structure. The ions in Fig. 3 that are unlabeled, in part, arose from other fragment ion series of *m/z* 620 or from dehydration of other ions.

Fluorescence Labeling of CEA-Producing Cells. To demonstrate that CEA is anchored to membranes with a phosphatidylinositol-glycan, the CEA-producing cell line LS 174T was treated with PtdIns-PLC and visualized by indirect immunofluorescence. Analysis by fluorescence flow cytometry (FACS) depicts a clear decrease in logarithmically displayed fluorescence intensity of PtdIns-PLC-treated cells relative to untreated cells (Fig. 4). Average decrease in fluorescence of the treated cells was calculated at 61%. This decrease in fluorescence was also seen under a fluorescence microscope (data not shown). NIH 3T3 cells, which do not express CEA, showed no significant reduction in fluorescence intensity upon PtdIns-PLC treatment (data not shown). Nor were any appreciable differences in the scatter curves of the PtdIns-PLC-treated and -untreated LS 174T cells seen, consistent with the conclusion that cell surface properties were unaffected by PtdIns-PLC treatment.

DISCUSSION

The membrane location of CEA on normal colon epithelia and colorectal adenocarcinoma is well established (1). The predicted amino acid sequence of CEA (3) includes a hydrophobic COOH-terminal stretch of 26 amino acids (residues 643–668), which is postulated as the membrane-anchoring

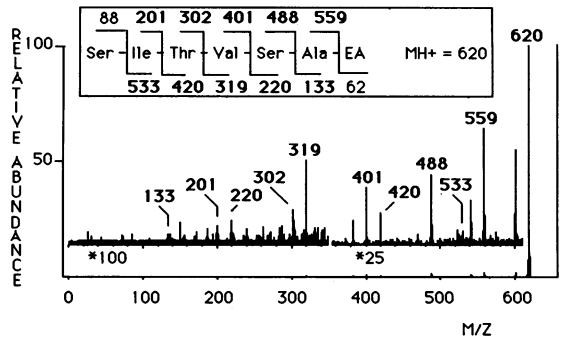

FIG. 3. B/E-linked scan mass spectrum for the natural tryptic peptide T12 (MH^+ = *m/z* 620). Insert defines the NH_2-terminal and COOH-terminal ion series of *m/z* 620. EA, ethanolamine. The spectrum is magnified 25× or 100× in the regions following the respective asterisk.

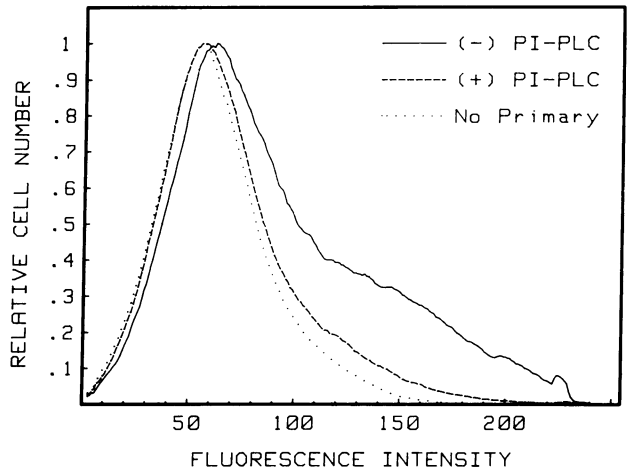

FIG. 4. Fluorescence flow cytometry of LS 174T cells. LS 174T cells were incubated with and without PtdIns-PLC and labeled with anti-CEA monoclonal antibody T84.1 followed by fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$. The cells were analyzed by fluorescence flow cytometry (FACS). Fluorescence intensity is displayed on a logarithmic scale. Background fluorescence (···) was measured by labeling the cells with the fluorescein isothiocyanate-conjugated antibody only. —, Cells untreated with PtdIns-PLC; ---, cells treated with PtdIns-PLC.

component for CEA. Because protein-sequence analysis of CEA provided no evidence for a hydrophobic COOH-terminal domain, we concluded that this domain was missing from the mature protein. Comparisons of these features at the COOH-terminus with proteins believed to be anchored to membranes with an ethanolamine-phosphatidylinositol-glycan complex suggested that CEA could be anchored similarily. Evidence for this type of anchoring structure in CEA was provided by the isolation and structural analysis of a peptide (T12) containing a covalently linked ethanolamine. This peptide is the terminal hexapeptide (residues 637–642) of the third immunoglobulin-like domain, a region in CEA immediately adjacent to the predicted COOH-terminal domain. The structure of the ethanolamine-linked peptide was determined by a combination of microsequence and FABMS analyses and was verified by comigration on reverse-phase HPLC of the natural product and a synthetic peptide of identical composition.

Recent studies on the structure of the phosphatidylinositol-glycan anchor show ethanolamine to be covalently coupled to the COOH-terminal residue of the protein (29–32). The protein is attached to the phosphatidylinositol-glycan anchor through a phosphodiester linkage between the ethanolamine and glycan portion of the complex. In the present studies, CEA was deglycosylated with HF, a procedure expected to hydrolyze the phosphodiester linkage; such treatment explains the missing remainder of the membrane anchor from peptide T12. To demonstrate that CEA was anchored with a phosphatidylinositol-glycan complex, we cleaved CEA from the membranes of colonic carcinoma cells with PtdIns-PLC. Analysis of fluorescence flow cytometry showed 61% reduction in the fluorescence intensity of PtdIns-PLC-treated cells relative to untreated cells. We conclude that CEA is processed posttranslationally to remove the 26-residue COOH-terminal domain with subsequent attachment of the ethanolamine-phosphatidylinositol-glycan complex to Ala-642.

The biological function of CEA is unknown, but the high level of CEA expression in fetal gut and colorectal carcinomas suggests a role in differentiation. Such a role is supported by the expression of CEA in tumors of tissues that would not normally produce it—namely, carcinomas of the breast, lung, ovary, pancreas, and prostate (1). Recent molecular cloning and protein studies demonstrate that CEA belongs to a multigene family including at least ten related glycoproteins and that it possesses three homologous domains related to immunoglobulin (2). Extensive immunologic and protein studies have further shown that each glycoprotein is membrane-anchored and differentially expressed in a variety of tissues (1). Each member of the family may be tissue or cell-specific or gene expression may be controlled in a tissue-specific manner. Occurrence of one or more immunoglobulin-like domains in these glycoproteins may have functional significance.

CEA is related structurally to Thy-1 and neural cell adhesion molecule (N-CAM) (2), two members of the immunoglobulin family anchored to membranes through a phosphatidylinositol-glycan complex (16, 32–35). Note that although N-CAM is expressed in three forms during development, only one of these forms contains the phosphatidylinositol-glycan complex (16). Changes in membrane anchoring and release of membrane-bound N-CAM are believed to be of functional importance (16). Unlike N-CAM, there is no evidence for alternative splicing in CEA, and we believe that the phosphatidylinositol-glycan-containing form is the only form expressed. Increased levels of CEA found in sera of cancer patients may be from membrane release of CEA or some defect in the phosphatidylinositol complex. Structural studies on the serum form of CEA would help distinguish between these two possibilities and provide insight into the release mechanism.

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