Evidence for Carboxyl-terminal Processing and Glycolipid-anchoring of Human Carcinoembryonic Antigen*

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We have investigated the post-translational modification of carcinoembryonic antigen (CEA) for membrane-anchoring in QGP-1 cells derived from a human pancreatic carcinoma. Pulse-chase experiments with [3H]leucine demonstrated that CEA was initially synthesized as a precursor form with M, 150,000 having N-linked high-mannose-type oligosaccharides, which was then converted to a mature form with M, 200,000 containing the complex type sugar chains. The mature protein thus labeled was found to be released from the cell surface by treatment with phosphatidylinositol-specific phospholipase C, suggesting that CEA is a phosphatidylinositol-linked membrane protein. This was confirmed by metabolic incorporation into CEA of 3H-labeled compounds such as ethanolamine, myo-inositol, palmitic acid, and stearic acid. The 3H-labeled fatty acids incorporated were specifically removed from the protein by nitrous acid deamination as well as by phosphatidylinositol-specific phospholipase C treatment. Since the available cDNA sequence predicts that CEA contains a single methionine residue only in its carboxyl-terminal hydrophobic domain, processing of the carboxyl terminus was examined by pulse-chase experiments with [35S]methionine. It was found that CEA with M, 150,000 was initially labeled with [35S]methionine but its radioactivity was immediately lost with chase. Taken together, these results suggest that CEA is anchored to the membrane by simultaneously occurring proteolysis of the carboxyl terminus and replacement by the glycosphospholipid immediately after the synthesis.

An increasing number of membrane proteins have been shown to possess covalently attached glycosphospholipid membrane anchors (Low, 1987; Cross, 1987). Involvement of phosphatidylinositol (PI) in such a unique anchoring was initially shown by pancreatic carcinoma. Pulse-chase experiments with [3H]leucine demonstrated that CEA was initially synthesized as a precursor form with M, 150,000 having N-linked high-mannose-type oligosaccharides, which was then converted to a mature form with M, 200,000 containing the complex type sugar chains. The mature protein thus labeled was found to be released from the cell surface by treatment with phosphatidylinositol-specific phospholipase C, suggesting that CEA is a phosphatidylinositol-linked membrane protein. This was confirmed by metabolic incorporation into CEA of 3H-labeled compounds such as ethanolamine, myo-inositol, palmitic acid, and stearic acid. The 3H-labeled fatty acids incorporated were specifically removed from the protein by nitrous acid deamination as well as by phosphatidylinositol-specific phospholipase C treatment. Since the available cDNA sequence predicts that CEA contains a single methionine residue only in its carboxyl-terminal hydrophobic domain, processing of the carboxyl terminus was examined by pulse-chase experiments with [35S]methionine. It was found that CEA with M, 150,000 was initially labeled with [35S]methionine but its radioactivity was immediately lost with chase. Taken together, these results suggest that CEA is anchored to the membrane by simultaneously occurring proteolysis of the carboxyl terminus and replacement by the glycosphospholipid immediately after the synthesis.

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An increasing number of membrane proteins have been shown to possess covalently attached glycosphospholipid membrane anchors (Low, 1987; Cross, 1987). Involvement of phosphatidylinositol (PI) in such a unique anchoring was initially suggested by release of alkaline phosphatase from membranes with bacterial PI-PLC (Ikezawa et al., 1976; Low and Finean, 1983; Yamashita et al., 1987) of CEA have been extensively studied, little was known about its primary structure until recently. Most recently, however, Oikawa et al. (1987) have reported the primary structure of CEA deduced from the cDNA sequence, demonstrating that CEA is synthesized as a precursor with a signal peptide followed by 668 amino acids of the putative mature molecule; the first 108 N-terminal residues are followed by three very homologous repetitive domains of 178 residues each and then by 26 mostly hydrophobic residues at the C terminus, which are suggested to comprise a membrane anchor. The presence of such a short C-terminal hydrophobic peptide sequence, however, led us to investigate possible involvement of the glycosphospholipid anchor in the mature CEA molecule after proteolytic processing of the C terminus. In this paper we describe the biosynthesis and post-translational modification of CEA in QGP-1 cells derived from a human pancreatic carcinoma, demonstrating that CEA is indeed anchored in the membrane by glycosphospholipid.
**Experimental Procedures**

**Materials**—L-[4,5-3H]Leucine (60.0 Ci/mmol), L-[35S]methionine (1200 Ci/mmol), L-[3H]cysteine (600 Ci/mmol), [9,10-3H]palmitic acid (25 Ci/mmol), [2-3H]inositol (17.1 Ci/mmol) were obtained from Du Pont-New England Nuclear. [3H-2]Ethanolamine (13.0 Ci/mmol) was obtained from Amersham Corp.; tunicamycin from Sigma; Pansorbin (fixed Staphylococcus aureus) cells from Calbiochem; Endo H from Seikagaku Kogyo (Tokyo, Japan); RPMI 1640 medium and Eagle’s minimum essential medium (MEM) from Nissui Seiyaku (Tokyo, Japan). PI-PLC was purified from Bacillus cereus as described previously (Komina et al., 1985). Polyclonal rabbit anti-human CEA antisera were prepared as described by Matsukata et al. (1978).

**Cells and Culture Conditions**—QGP-1 cells derived from human pancreatic carcinoma (Kaku et al., 1988) were cultured at 37°C in 60-mm Falcon dishes containing RPMI 1640 medium supplemented with fetal calf serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamycin (50 μg/ml). Prior to each experiment, the cells were pretreated for 4 days with 2 mM sodium butyrate for the induction of CEA (Tsao et al., 1983). Sodium butyrate (2 mM) was previously (Takami et al., 1988). Unless otherwise specified, the cells were harvested, lysed, and centrifuged at 12,000 g for 3 min. The cell lysates were mixed with a mixture of protease inhibitors (antipain, elastatinal, leupeptin, chymostatin, pepstatin A, and trasylof, finally 10 μg each/ml), sonicated for 2 min, and centrifuged at 15,000 × g for 30 min (Takami et al., 1988). The resultant supernatants were used for immunoprecipitation of CEA.

**Other Metabolic Labeling Procedures**—Unless otherwise indicated, cells were incubated at 37°C for 16–18 h with either [3H]inositol (0.5 μCi/ml dish), [3H]ethanolamine (0.5 μCi/ml dish), [3H]palmitic acid (2.5 μCi/ml dish), or [3H]methionine (200 μCi/1.5 ml dish) was carried out following preincubation of cells in the MEM lacking unlabeled methionine. At the indicated times of chase, the cells were separated from the medium, washed twice with Dulbecco’s phosphate-buffered saline, and lysed in 0.5 ml of the same buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.5% SDS, boiled for 3 min, and centrifuged at 12,000 g for 3 min. To each supernatant, 8 μl of a 10% (w/v) suspension of Pansorbin (prepared by precipitation of rabbit serum), and the mixtures were boiled for 3 min, centrifuged at 12,000 g for 3 min. The resultant supernatants were used for immunoprecipitation of CEA.

**Immunoprecipitation of Labeled CEA**—A 3-μl aliquot of preimmune rabbit serum was added to each cell lysate, and the mixtures were incubated at 4°C for 1 h. The samples were treated for 1 h with shaking at 4°C with 100 μl of a 10% (w/v) suspension of Pansorbin and centrifuged for 3 min at 12,000 × g (Takami et al., 1988). The supernatants were then incubated with 3 μl of anti-CEA serum for 4 h at 4°C and then overnight at 4°C with Pansorbin as above. The Pansorbin immunocomplex was then extensively washed as described previously (Takami et al., 1988). Unless otherwise specified, the washed immunoprecipitates were finally suspended in 20–60 μl of 62.5 mM Tris-HCl (pH 6.8) containing 1% SDS and 1% 2-mercaptoethanol (solubilizing buffer). The samples were boiled for 3 min, centrifuged at 12,000 × g for 10 min to remove Pansorbin, and stored at −20°C until use.

**Enzyme Digestion**—The washed Pansorbin immunocomplex was suspended in 50 μl of 50 mM acetate buffer (pH 5.5) containing the protease inhibitors (10 μg each/ml) and incubated with Endo H (final concentration 2 units/ml) at 37°C for 16 h (Mismi et al., 1986). The digested samples were washed with 62.5 mM Tris-HCl (pH 6.8), resuspended in the solubilizing buffer, and treated for solubilization as above. For treatment with PI-PLC, the Pansorbin immunocomplex was suspended in 50 μl of 50 mM Tris-HCl (pH 7.5) containing 1% Triton X-100, 0.5% deoxycholate, and 0.5% SDS, boiled for 3 min, and then centrifuged at 12,000 × g for 3 min. The cells were washed twice with MEM and reincubated at 37°C with 30 μl of PI-PLC (10 μg/ml) in 1 ml of MEM. At the indicated times, cells were scraped from each dish, and the cell suspensions were centrifuged at 12,000 × g for 10 min for separation of medium from cells, which were lysed in 0.5 ml of the lysis buffer. The medium and cell lysates thus prepared were used for immunoprecipitation of CEA as described above.

**Nitrous Acid Deamination**—Immunoprecipitates were resuspended in 50 μl of 5 mM Tris-HCl (pH 7.5) containing 1% SDS, boiled for 3 min, and centrifuged as above. The resulting supernatants were diluted to 10-fold with 0.25 M sodium acetate (pH 3.5) containing 0.2 M NaNO2, and the mixtures were incubated at 50°C for 4 h (Shively and Conrad, 1976). The reaction was terminated by precipitating the protein with 0.5 ml of 2% phosphotungstic acid/1 M HCl after addition of carrier hemoglobin (50 μg) into each sample. The precipitates were washed with the same acid solution and dissolved in 60 μl of the solubilizing buffer followed by neutralization with 3 M Tris-HCl (pH 7.5).

**SDS-PAGE and Fluorography**—SDS-PAGE was carried out in slab gels with 7.5% acrylamide according to Laemmli (1970), followed by fluorography (Bönné and Laskey, 1974). Apparent molecular weights were determined by coelectrophoresis of marker proteins metabolically labeled with [35S]methionine (Misman et al., 1986; Oda et al., 1987); rat pro-C3 (M, 180,000), C3 α chain (115,000), transferrin (77,000), C3 β chain (65,000) and α1-protease inhibitor (56,000). In some experiments, the radioactive areas detected by fluorography were excised from gels, solubilized in Protosol, and counted for the radioactivity by a scintillation counter (Oda et al., 1978).

**Results**

**Biosynthesis and Processing of CEA—**QGP-1 cells were pulse-labeled with [3H]leucine for 30 min and then chased. Fig. 1 shows sodium dodecyl sulfatopolycarboxylic acid gel electrophoresis (SDS-PAGE)/fluorography of immunoprecipitates of CEA. CEA was shown to be initially synthesized as a form with M, 150,000 (Fig. 1A, lane 1), which was then processed to a larger form with M, 200,000 (lanes 2–5). Upon treatment with Endo H, the M, 150,000 form was converted to a much smaller form with M, 82,000 (Fig. 1B, lanes 1 and 2), whereas the M, 200,000 form was unchanged (lanes 3–5). CEA prepared from tunicamycin-treated cells (TM) migrated to a position with M, 75,000. These results indicate that CEA having the polypeptide chain with M, 75,000 is synthesized as the precursor with high-mannose-type oligosaccharides (M, 150,000) and processed to the mature form with the complex types (M, 200,000) during intracellular transport to the cell surface. The difference in molecular mass between the Endo H-treated form and the tunicamycin-treated one may be due to asparagine-linked GlcNAc residues in the form that was not digested with Endo H.

**FIG. 1.** Pulse-chase labeling of CEA with [3H]leucine. Cells were pulse-labeled with 100 μCi of [3H]leucine for 30 min and chased. At the indicated times, cells were harvested, lysed, and used for immunoprecipititation of CEA. The immunoprecipitates before (A) and after (B) Endo H treatment were analyzed by SDS-PAGE/fluorography as described under “Experimental Procedures.” Lane 1, no chase; lanes 2-5, chased for 30 min, 1 h, 2 h, and 3 h, respectively. M, molecular markers including rat pro-C3 (M, 180,000), C3 α chain (115,000), transferrin (77,000), C3 β chain (65,000) and α1-protease inhibitor (56,000) which had been metabolically labeled with [35S] methionine. TM, [3H]leucine-labeled CEA obtained from the tunicamycin-treated cells.
even after the treatment, since CEA has 28 potential glycosylation sites (Oikawa et al., 1987).

**Metabolic Labeling of CEA with Glycolipid Components**—Fig. 2 shows metabolic incorporation of possible glycolipid components into CEA. Incubation of cells for 16-18 h with [3H]leucine-labeled ethanolamine, myo-inositol, stearic acid, and palmitic acid (Fig. 2A, lanes 2-5, respectively) followed by immunoprecipitation yielded a single labeled form corresponding to the mature form of [3H]leucine-labeled CEA (lane 1). In the presence of tunicamycin (Fig. 2B), the unglycosylated form with M, 75,000 was also labeled with [3H]labeled ethanolamine (lane 2) and stearic acid (lane 3). The results indicate that CEA is modified with these glycolipid components in both the absence and presence of tunicamycin, in contrast to its N-linked glycosylation.

**Release of Fatty Acids from CEA**—CEA labeled with various compounds was digested with PI-PLC and analyzed by SDS-PAGE/fluorography (Fig. 3A). The [3H]leucine- or ethanolamine-labeled CEA showed no significant change in SDS-PAGE before and after treatment with the enzyme (lanes 1-4). The radioactivity of [3H]stearic acid, however, was completely removed from the protein by treatment with PI-PLC (lane 6). The same results were obtained with [3H]palmitate-labeled CEA (data not shown). Since PI-PLC used here is strictly specific for phosphatidylinositol as substrate (Komnami et al., 1985), the results indicate that the [3H]-labeled fatty acids incorporated comprise a phosphatidylinositol moiety together with other components in the molecule.

The [3H]-labeled CEA was then treated with nitrous acid which is known to cleave glycosidic linkage of glucosamine residues with a free primary amino group (Shively and Conrad, 1976). As shown in Fig. 3B, the treatment completely removed [3H]-labeled stearic acid from the protein (lane 4), in contrast to its effect on the [3H]leucine-labeled CEA (lane 2). The results indicate possible involvement of glucosamine in linkage of the lipid components to CEA. Taken together, these results (Figs. 2 and 3) suggest that the components characterized above are covalently linked as a phosphatidylinositol glycolipid to the CEA molecule.

**Release of CEA from Cell Surface with PI-PLC**—We further examined involvement of phosphatidylinositol in membrane-anchoring of CEA. Cells were pulse-labeled with [3H]leucine and chased for 4 h so that most of the [3H]-labeled CEA could reach the cell surface. The cells were then incubated at 37°C in the presence or absence of PI-PLC up to 4 h. As illustrated in Fig. 4, the labeled CEA was rapidly released from the cells in the presence of PI-PLC, and at the end of incubation (4 h) the released CEA in the medium reached about 80% of the protein initially associated with cells. In contrast, no significant amount of the protein appeared in the medium in the absence of PI-PLC. Such a specific release of CEA by PI-PLC suggests that the protein is anchored to the cell surface by phosphatidylinositol.
were exposed for 30 min; and chased. At the indicated times, cells were harvested, lysed, and used for immunoprecipitation of CEA, followed by SDS-PAGE/fluorography as described under "Experimental Procedures." Lanes 1–3 show the \[^{[3]H}\]leucine-labeled samples and lanes 4–6 represent the \[^{[35]S}\]methionine-labeled ones; lanes 1 and 4, no chase; lanes 2 and 5, chased for 30 min; and lanes 3 and 6, chased for 90 min. Lanes 1–3 were exposed for 4 days and lanes 4–6 for 20 days.

Post-translational Processing of the Carboxyl Terminus—A cDNA sequence predicts that CEA contains a hydrophobic peptide domain at the carboxyl terminus which would participate in membrane localization (Oikawa et al., 1987). The domain contains a methionine residue at the 11th position from the carboxyl terminus which is 1 of 2 methionine residues identified in the whole primary structure of CEA precursor; the other is found as the first residue of its signal peptide which should be cotranslationally cleaved off. Thus, CEA contains a single methionine residue at the carboxyl-terminal region after the completion of its translation.

On the basis of these findings, we carried out pulse-chase experiments using \[^{[35]S}\]methionine as a tracer (Fig. 5). The \[^{35}S\]-labeled CEA was indeed identified as a molecule with \(M_\text{r} = 150,000\) (lane 4) in the cells which had been labeled for 10 min. The results are in contrast to those obtained with \[^{[3]H}\]leucine under the same conditions as above (Fig. 5, lanes 1–3) and under other conditions (Fig. 1A). The incorporation of \[^{[35]S}\]methionine into CEA followed by its disappearance suggests that the protein is initially synthesized as a precursor with the carboxyl-terminal hydrophobic peptide extension, which is cleaved off from the protein immediately after its synthesis.

DISCUSSION

The cDNA translated sequence of CEA contains a hydrophobic carboxyl terminus which could act as a membrane anchor although it lacks a cytoplasmic tail (Oikawa et al., 1987). The presence of such a characteristic carboxyl terminus has been identified in African trypanosome VSG (Cross, 1984), Thy-1 (Seki et al., 1985), alkaline phosphatase (Millán, 1986; Misumi et al., 1988), and decay-accelerating factor (DAF) of complement (Caras et al., 1987a; Medof et al., 1987), and demonstrated to have a close relationship with their post-translational modification by the glycosylphospholipid for membrane-anchoring; VSG (Ferguson et al., 1985b), Thy-1 (Tse et al., 1985; Low and Kincade, 1985), alkaline phosphatase (Low and Zilversmit, 1980; Takami et al., 1988), and DAF (Davitz et al., 1985; Medof et al., 1986). These relationships suggest the possibility that CEA is also a glycosylphospholipid-anchored membrane protein. All the data presented here support this prediction. CEA was metabolically labeled with the radioactive compounds characteristic for the glycolipid components such as ethanolamine, inositol, palmitic acid, and stearic acid and was released from the cell surface by PI-PLC. The presence of glucosamine in the linkage was suggested by nitrous acid deamination (Fig. 3B), as observed for other proteins (Ferguson et al., 1985b; Medof et al., 1986; Takami et al., 1988).

The glycolipid modification is considered to require prior proteolytic cleavage of the hydrophobic carboxyl terminus (Cross, 1987; Low, 1987). The incorporation of \[^{[35]S}\]methionine into CEA with \(M_\text{r} = 150,000\) indicates that the protein is initially synthesized as a precursor with the predicted C-terminal extension containing a methionine residue (Fig. 5). The immediate removal of the extension was demonstrated by disappearance of the \[^{35}S\]-labeled CEA during chase. This is in contrast with the \[^{[3]H}\]leucine-labeled form, most of which remained as the \(M_\text{r} = 150,000\) form (lane 2). Thus, it is likely that the remaining form, although having the same molecular mass as that of the precursor, is already proteolytically processed and immediately modified with the glycolipid anchor. In fact, this form with \(M_\text{r} = 150,000\) was also labeled with the \(^3\text{H}\)-labeled glycolipid components as faintly observed in Fig. 2A and more intensely under appropriate conditions (data not shown). No significant difference in molecular mass between the precursor and modified forms may indicate that a mass of the peptide cleaved (at most 26 amino acid residues, Oikawa et al., 1987) is close to that of the newly attached glycolipid anchor; a small difference, if any, could not be detected in such large molecules with \(M_\text{r} = 150,000\) under the conditions used.

Tawaragi et al. (1988) have recently reported the primary structure of nonspecific cross-reacting antigen (NCA), a member of CEA gene family, deduced from the cDNA sequence. The cDNA encodes a precursor form of a polypeptide consisting of a 34-residue signal sequence, a 108-residue N-terminal domain, a 178-residue domain, and a 24-residue domain rich in hydrophobic amino acids at the carboxyl terminus. The presence of the hydrophobic C-terminal domain suggests that NCA is also a glycolipid-anchored protein. Our preliminary experiments have demonstrated that NCA is labeled with \[^{[3]H}\]ethanolamine and \[^{[3]}\text{H}\]stearic acid (data not shown). It is of interest to know whether all the CEA-related antigens have the same system for membrane-anchoring. Most human carcinoma cell lines producing CEA also synthesize NCA (Ichiki et al., 1986). Although CEA and NCA are synthesized as the membrane-bound proteins in most of these cells examined, some cell lines, for instance CCK-81 derived from colon carcinoma, produce both the antigens most of which are released into the culture medium like a secretory protein (Ichiki et al., 1986). One of possible mechanisms for their release is that the antigens are released into the culture medium like a secretory protein (Ichiki et al., 1986). One of possible mechanisms for their release is that the antigens are released into the culture medium like a secretory protein (Ichiki et al., 1986).
The signal that dictates processing of the carboxyl terminus and attachment with the glycolipid is not known. It is, however, believed that the presence of a hydrophobic C-terminal domain without a cytoplasmic tail plays the most important role in the unique post-translational modification of these proteins by the glycolipid. This is strongly supported by most recent evidence that the last 37 amino acids of membrane DAF, when fused to the carboxyl terminus of a secretory protein, are sufficient to target the fusion protein to the plasma membrane by means of a glycolipid anchor (Caras et al., 1987b).

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