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CEA Is the Major PHA-L-Reactive Glycoprotein in Colon Carcinoma Cell Lines and Tumors: Relationship between K-*ras* Activation and β1–6 Branching of N-Linked Carbohydrate on CEA

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Previously we have shown that a positive correlation existed between the presence of β1–6 branching of N-linked carbohydrate (detected as PHA-L reactivity) and the level of Ras activation in colon carcinoma cell lines. In these cell lines the major PHA-L-reactive species was found to be 180 kDa. Here we identified this species to be carcinoembryonic antigen (CEA) by demonstrating that: (a) CEA immunoreactivity and PHA-L reactivity colocalized on blots of crude cellular membranes from these cell lines, and that (b) immunoprecipitation of CEA resulted in quantitative coprecipitation of PHA-L reactivity at 180 kDa. Metabolic labeling of cell line HTB39 with [^{3}H]mannose revealed that CEA was the predominantly labeled glycoprotein. This indicated that CEA was the major PHA-L-reactive species due its high level of expression. The amount of PHA-L reactivity present on CEA, expressed as the PHA-L/CEA ratio, was found to vary between cell lines. This ratio was found to correlate closely with the level of Ras activation in these cells. In cellular membrane isolated from primary colon carcinoma, the major PHA-L-reactive species was also 180 kDa. This reactivity colocalized with CEA immunoreactivity, indicating that the major β1–6-branching glycoprotein in membranes from primary colon carcinoma was CEA. Similar to that seen in cell lines, the amount of PHA-L reactivity on CEA in human tumor samples varied, suggesting that a similar paradigm of Ras-induced expression of β1–6 branching may occur in human colon carcinoma.

Numerous changes in glycosylation have been observed in transformed cells. These changes include an increase in sialylation and the addition of complex branching of N-linked oligosaccharides; both of which have been linked to a more aggressive or metastatic phenotype (1–4).

Generally, investigators have documented the above changes in glycosylation in cells transformed by the expression of oncogenes. Most studied were cell lines transfected with oncogenic forms of *ras* and *src*. In both cases, cells expressing these oncogenes showed an increase in sialylation (5) and the addition of β1–6 branching at the trimannosyl core of N-linked carbohydrate (2, 6–8). Phytohemagglutinin–leukoagglutinin (PHA-L) is a lectin that recognizes the β1–6 branch in N-linked carbohydrate (9). Increases in β1–6 branching have been shown to be attributable to elevations in β1–6 *N*-acetylglucosaminyltransferase V activity (8, 10–11).

p21Ras is a signaling protein with intrinsic GTPase activity and cycles between an inactive GDP-bound state and an activated GTP-bound state (12). The Ras GDP-GTP cycle is highly regulated, and in normal resting cells the vast majority of p21ras is bound to GDP. Activating mutations in K-*ras* at codons 12, 13, and 61 produce mutant proteins that have a compromised ability to hydrolyze GTP. Thus, expression of mutant forms of *ras* results in constitutive activation of the Ras-signaling pathway detected as an increase in the cellular Ras-GTP level. Between 30 and 40% of colon carcinomas have been found to harbor activating mutations in K-*ras* (13–14).

We have previously found an association between the extent of Ras-activation and the amount of β1–6 branching of N-linked carbohydrate in colon (15) and pancreatic (16) cell lines. Colon carcinoma cell lines with high levels of PHA-L reactivity were found to have, as their major PHA-L reactive species, a 180-kDa glycoprotein. In this report we identified this species to be CEA. We further show that the amount of PHA-L

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reactivity on CEA in these cell lines was correlative with cellular Ras-GTP levels. This was consistent with previous data showing a correlation between the amount of PHA-L reactivity on CEA and the amount of K-Ras present in PHA-L resistant cells of colon carcinoma cell line HTB39 (17). Lastly, like that seen in cell lines, we found that CEA was the major PHA-L reactive species in cellular membranes derived from primary colon carcinoma and that the amount of PHA-L on CEA varied widely.

MATERIALS AND METHODS

Cell culture. All cell lines were grown in RPMI with 5% (v/v) fetal bovine serum (FBS), except CaCo-2 which was grown in MEM with 10% (v/v) FBS.

K-ras genotyping and Ras-GTP analysis. Genotyping of the first and second exon of the K-*ras* gene and Ras-GTP levels were determined as described previously (15). Cell lines and tumor samples were fractionated (see below) and a small amount of the nuclear fraction was boiled in sterile water followed by centrifugation at 16,000*g* for 10 min. The supernatant, containing liberated cellular DNA, was used for amplification of the K-*ras* gene. Polymerase chain reaction of the first and second exon of the K-*ras* gene employed amplification of 35 cycles using primers that bordered the exon of interest. The resulting DNA product was sequenced directly and resolved on a 10% polyacrylamide gel.

Cell membrane preparation and lectin blot analysis. Total cellular membranes from cell lines and tumor samples were prepared and analyzed by lectin blotting as described previously (15). In summary, cells were harvested by scraping, washed once in phosphate buffered saline (PBS), and stored as cell pellets at −80°C. Cell pellets were then processed as follows: pellets were quickly thawed, suspended in 500 μl of PBS with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM ethylenediaminetetraacetic acid (EDTA). They were then dounce homogenized with 60 strokes of a tight pestle, and centrifuged at 600*g* for 5 min. The nuclear enriched pellet was used as starting material for amplification of the K-*ras* gene using polymerase chain reaction (see above). The supernatant was centrifuged at high speed to harvest total cellular membrane. Membrane protein was solubilized in PBS with 1% NP40, 2 mM (EDTA), 1 mM (PMSF), 10 μg/ml of each aprotinin and leupeptin and 5 μg/ml of pepstatin (solubilization buffer). Total protein in membrane samples was determined using the BCA protein assay (Pierce Chemical Co., Rockford, IL). Tumor samples were pulverized in liquid nitrogen, suspended in PBS with protease inhibitors, homogenized by douncing, and processed in an identical fashion as that described for cell lines. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transfer of protein to nitrocellulose membrane, and lectin blotting was performed as described previously (15).

Immunoblotting and immunoprecipitation of CEA. A polyclonal antibody to CEA was used in all experiments. For immunoblotting, nitrocellulose membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 with 3% BSA (w/v) for 4 h. The blot was probed for 1 h with a 1:2000 dilution of anti-CEA (The Binding Site, San Diego, CA), washed and incubated for 1 h with a 1:3000 dilution of a horseradish peroxidase (HRP)-conjugated secondary antibody. Bound antibody was detected by chemiluminescence.

For immunoprecipitation of CEA, solubilized membrane protein was incubated with the indicated amount of anti-CEA overnight at 4°C on a mutator. CEA antibody was then harvested by the addition of affinity purified rabbit anti-sheep IgG bound to Protein A Sepharose. After an hour incubation, the resulting immunoprecipitate was harvested by brief centrifugation at 2000*g*. The supernatant was removed and placed immediately in SDS–PAGE loading buffer with β-mercaptoethanol. The pellet was washed four times in solubilization buffer and the immune complexes liberated and dissociated in gel loading buffer containing β-mercaptoethanol. The immunoprecipitate and supernatant in gel loading buffer was placed at 100°C for 2 min and then separated on either a 7 or 8% SDS–PAGE gel. For autoradiography of labeled material, the gel was fixed, placed in Entensify (New England Nuclear, Boston, MA), dried and exposed to autoradiographic film at −80°C for 30 days. For lectin or antibody membrane blot analysis, the contents of the gel were transferred to nitrocellulose membrane and probed accordingly.

Metabolic labeling of cell line HTB39. Cells were labeled with 10 μCi/ml of ^{3}H]mannose (20-30 Ci/mmol, New England Nuclear, Boston, MA) for 48 h. After labeling the cells were harvested, centrifuged, and quickly frozen on dry ice. Cell pellets were stored at −80°C. The cell pellet was defrosted and crude cellular membranes prepared as described above. Five μg of solubilized membrane protein (approximately 2.5×10^4 cpm) was used for autoradiographic analysis or immunoprecipitation studies with anti-CEA antibody.

RESULTS

The major PHA-L-reactive species in colon carcinoma cell lines is CEA. Previously, the major PHA-L reactive glycoprotein in colon carcinoma cell lines was shown to be a species of 180 kDa (15). Since CEA is a highly glycosylated protein of approximately 180 kDa, and is frequently overexpressed in colon carcinomas, we wanted to determine whether this species was CEA.

First, PHA-L lectin blotting and CEA immunoblotting were performed on crude membranes prepared from nine different cell lines derived from human colon carcinoma. This analysis was performed to ascertain if PHA-L reactivity and CEA immunoreactivity co-localized at the same molecular weight. PHA-L lectin and CEA antibody reactivities were compared directly by sequential probing of the same blot. As shown in Fig. 1, six of the nine cell lines had both negligible PHA-L lectin and CEA antibody reactivity at 180 kDa. These cell lines represented three K-*ras* wild-type cell lines (Colo205, SW1417, and CaCo-2), two cell lines with K-*ras* codon 13 mutations (DLD1 and HCT15) and one K-*ras* codon 12 mutant (SW620). The seventh cell line, LS180, a K-*ras* codon 12 mutant, had detectable CEA immunoreactivity but little corresponding PHA-L reactivity at 180 kDa. In the two remaining lines that had K-*ras* codon 12 mutations (SW1116 and HTB39), PHA-L reactivity at 180 kDa and CEA immunoreactivity colocalized. The concordance between the localization of CEA immunoreactivity and PHA-L reactivity suggested that the PHA-L-reactive species at 180 kDa was CEA.

To confirm this hypothesis, solubilized membrane protein from cell line HTB39 was subjected to immunoprecipitation with varying amounts of anti-CEA and the resulting immunoprecipitates and supernatants were blotted and probed with PHA-L (Fig. 2). Use of 2 μl of polyclonal anti-CEA resulted in the immunoprecipitation of PHA-L reactivity at 180 kDa (top-left panel). Doubling the amount of CEA antibody in the immunoprecipitation reaction resulted in even more PHA-L reactivity associating with the immunoprecipitate (top-left panel), and a further decrease in the

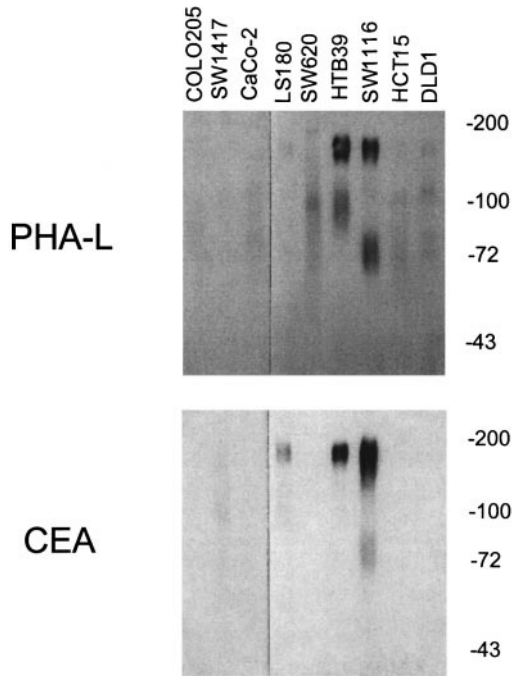

FIG. 1. Detection of PHA-L reactivity and CEA immunoreactivity in colon carcinoma cell lines. 2.5 μg of crude membrane protein from nine cell lines was separated on an 8% SDS–PAGE gel, transferred to nitrocellulose and probed first with PHA-L coupled to HRP (top). The blot was then stripped and probed with anti-CEA followed by incubation with a HRP-labeled secondary antibody (bottom). Bound lectin and CEA antibody were detected using chemiluminescence. At right, locations of molecular weight markers (in kDa).

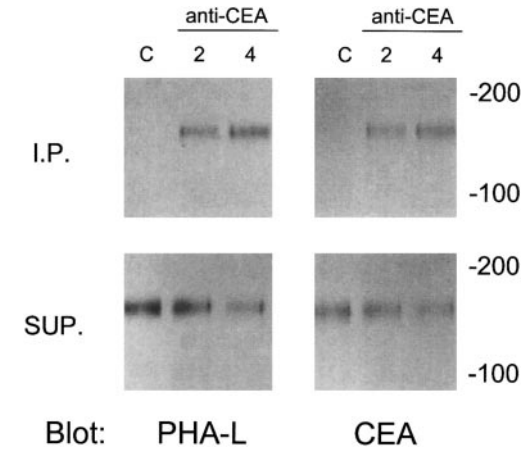

FIG. 2. Identification of PHA-L reactivity associated with CEA in cell line HTB39. Equal amounts of solubilized membrane protein from cell line HTB39 were immunoprecipitated with either 2 or 4 μl of anti-CEA antibody or 4 μl of normal sheep IgG (C). The immune complexes were harvested by incubation with rabbit anti-sheep IgG bound to protein A–Sepharose. The immunoprecipitates (IP) (top panels) and supernatants (SUP) (bottom panels) from each IP were harvested. Equal amounts of IP, and supernatant were separated on a 7% SDS–PAGE gel and transferred to nitrocellulose. The membrane was probed with PHA-L (left panel), stripped, and probed with anti-CEA (right panel). Bound lectin and antibody were detected as outlined in the legend to Fig. 1. At right, locations of molecular weight markers (in kDa).

residual amount of PHA-L reactivity in the supernatant (bottom-left panel). Stripping the blot and probing with anti-CEA (top-right and bottom-right panels) revealed that CEA and PHA-L reactivity cosegregate in both the immunoprecipitates and supernatants (compare left and right panels). Immunoprecipitation with nonimmune IgG resulted in neither PHA-L reactivity (top-left panel) or CEA immunoreactivity (top-right panel) associating with the immunoprecipitate. As a control for PHA-L reactivity, a direct comparison of equal amounts of immunoreactive, immunoprecipitated CEA from cell lines LS180 and HTB39 was performed. As seen in Fig. 3, CEA from HTB39 had significant PHA-L reactivity but CEA from LS180 had negligible PHA-L reactivity. This result demonstrated that PHA-L binding to CEA was selective and not attributable to nonspecific lectin-protein interactions. This result also indicated that the amount of PHA-L reactivity associated with CEA varied significantly between cell lines HTB39 and LS180 (see below). Collectively, these data confirm that CEA was the PHA-L-reactive species seen at 180 kDa.

High level of membrane-associated CEA protein is the mechanism by which CEA is the major PHA-L-reactive species. Since CEA overexpression is common in colon carcinoma, we investigated whether CEA was the major PHA-L reactive species present in the membranes of colon carcinoma cell lines by virtue of overexpression. To ascertain qualitatively the relative level of synthesis of CEA compared to other glycoproteins, cell line HTB39 was metabolically labeled with

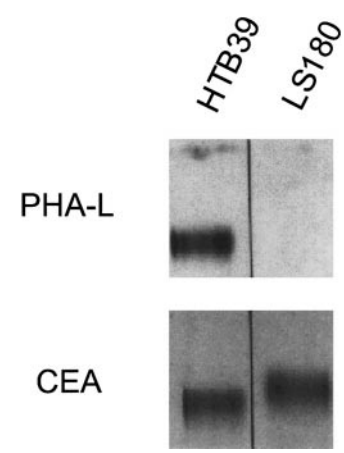

FIG. 3. Comparison of PHA-L reactivity associated with CEA in cell lines HTB39 and LS180. Equivalent amounts of immunoreactive CEA were immunoprecipitated from cell lines HTB39 (left lane) and LS180 (right lane) as described in Fig. 2. The immunoprecipitates were separated on a 7% SDS–PAGE gel and transferred to nitrocellulose. The nitrocellulose membrane was probed with PHA-L (top panel), stripped and then probed with anti-CEA (bottom panel). Bound probes detected as described in the legend to Fig. 1.

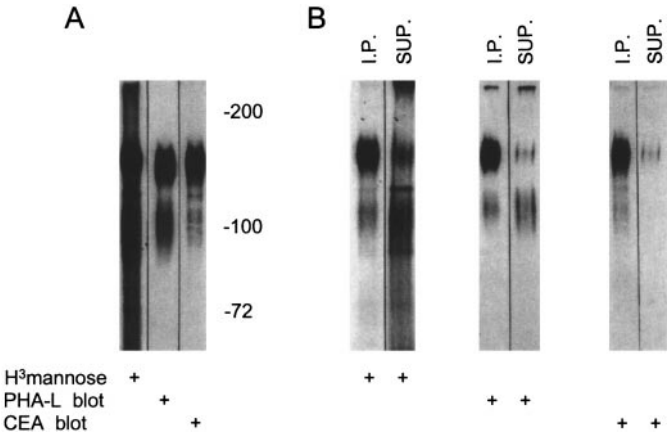

FIG. 4. Metabolic labeling and analysis of CEA from cell line HTB39. Cell line HTB39 was metabolically labeled with [^{3}H]mannose and membranes prepared. (A) 2.5 μg of labeled membrane was separated on a 7% SDS–PAGE gel and either fixed, dried and exposed to autoradiography (lane 1) or the contents were transferred to nitrocellulose and probed with either PHA-L (lane 2) or anti-CEA (lane 3). (B) An identical amount of labeled material was immunoprecipitated with anti-CEA and the resulting immunoprecipitate (IP) and supernatant (SUP) separated by SDS–PAGE and analyzed by either autoradiography (left panel), or the contents were transferred to nitrocellulose and probed sequentially with PHA-L (middle panel) or anti-CEA (right panel). Bound lectin and antibody was detected as described in the legend to Fig. 1. (A) Locations of molecular weight markers (in kDa).

[^{3}H]mannose so that glycoproteins would be labeled based upon rate of carbohydrate incorporation into newly synthesized protein. As an initial characterization, crude cellular membranes from labeled cells were prepared and analyzed by either autoradiography or blotting with anti-CEA and PHA-L (Fig. 4A). This analysis was performed to ascertain the molecular weight profile of glycoproteins labeled in HTB39 and to determine if a prominently labeled molecular weight species was detected at 180 kDa. The labeling profile of crude membrane protein from cell line HTB39 is shown in Fig. 4A, lane 1. The major mannose labeled species was 180 kDa followed by a less intense smear that extended from 100 to 150 kDa. Probing this material with PHA-L revealed PHA-L reactivity that colocalized with both these regions (Fig. 4A, lane 2). When probed with anti-CEA, immunoreactivity was seen predominantly at 180 kDa (Fig. 4A, lane 3). Thus, the prominently ^{3}H-labeled material at 180 kDa colocalized with both and PHA-L reactivity and CEA immunoreactivity.

To confirm that the 180-kDa labeled species was CEA, labeled membrane glycoprotein was immunoprecipitated with polyclonal anti-CEA antibody. The immunoprecipitate and corresponding supernatant were subjected to SDS–PAGE and analyzed by autoradiography. As seen in Fig. 4B (left panel), almost all of the 180 kDa labeled species was precipitated with anti-CEA antibody with only a modest amount of the 180 kDa labeled material left in the supernatant. Conversely, the majority of the 100 to 150 kDa labeled material was found in the supernatant with little in the immunoprecipitate. Probing nitrocellulose membrane containing a duplicate amount of immunoprecipitate and supernatant with PHA-L (Fig. 4B, middle panel) showed that essentially all of the PHA-L reactivity present at 180 kDa was found in the precipitate; only a minor fraction was found in the supernatant. The same was found when the blot was stripped and probed with anti-CEA (Fig. 4B, right panel). The finding that some CEA was still present in the supernatant suggested that the residual labeled material at 180 kDa in the supernatant represented CEA which was not immunoprecipitated. Collectively, this data indicated that CEA was the major PHA-L reactive glycoprotein present because of its level of expression in that it represented a significant amount of the total amount of labeled glycoprotein present in the membrane of cell line HTB39.

The results presented in Fig. 4B suggested that the labeled glycoprotein smear from 100 to 150 kDa in cell line HTB39 represented other glycoproteins rather than degradation products of CEA. In cell line SW620, where no CEA protein is detected, a similar PHA-L reactive smear was seen (Fig. 1). This indicated that CEA expression was not obligatory for the presence of the 100-150 kDa PHA-L reactive species in colon carcinoma cell lines. That both HTB39 and SW620 were sensitive to the cytotoxic effects of PHA-L (15) suggested that they possessed common PHA-L reactive targets that mediated sensitivity to PHA-L. Possibly, a reactive species present in the 100-150 kDa range conveyed PHA-L sensitivity to these cell lines.

Stoichiometry of β1–6 modification of CEA in cell lines: correlation with degree of Ras-activation. Comparison of PHA-L reactivity with CEA immunoreactivity in Fig. 1, and Fig. 3 indicated that the amount of PHA-L reactivity present on CEA varied significantly between cell lines. In comparing cell lines SW1116 and HTB39, there was more CEA immunoreactivity in cell line SW1116 than in HTB39; yet there was more PHA-L reactivity in HTB39 than SW1116. Furthermore, in cell line LS180 there was demonstrable CEA immunoreactivity but barely detectable PHA-L reactivity (Fig. 3). All three of these cell lines had mutations in K-*ras* at codon 12 and all had Ras-GTP levels higher than that seen in K-*ras* wild-type cell lines (15). Since an association between Ras-activation and β1–6 branching had been noted previously (15), it was questioned whether the amount of PHA-L reactivity on CEA could be related to the level of Ras-activation in these cell lines. To ascertain whether a correlation existed between the PHA-L reactivity present on CEA and the cellular Ras-GTP levels in these cells, the blots in Fig. 1 were analyzed by densitometry to determine

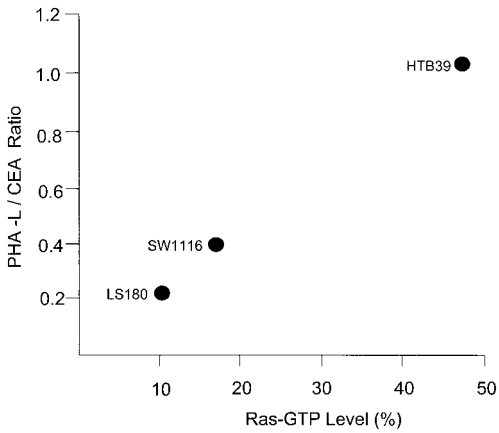

FIG. 5. Relationship of the CEA/PHA-L ratio to Ras activation in colon carcinoma cell lines. Autoradiographic films from Fig. 1 were scanned by laser densitometry and the signals generated at 180 kDa by anti-CEA and PHA-L reactivity assessed. A ratio of these values was calculated (PHA-L/CEA) for each cell line and plotted against the Ras-GTP level calculated for that cell line.

the relative intensities of the signals generated by PHA-L and anti-CEA. From this data, a ratio (PHA-L densitometry units/CEA densitometry units) was calculated for the three cell lines demonstrating CEA immunoreactivity. The resulting PHA-L/CEA ratios were plotted against the calculated Ras-GTP level determined for these cell lines. A positive correlation was seen between the Ras-GTP level and PHA-L/CEA ratio calculated for the three cell lines (Fig. 5). This result confirmed that a correlation existed between the PHA-L/CEA ratio and Ras-GTP level in these cell lines.

CEA is the major PHA-L-reactive glycoprotein in colon carcinoma. Cellular membranes were prepared from tumor and adjacent normal mucosa from three patients who underwent surgery for resection of primary colon carcinoma. These membranes were analyzed by PHA-L lectin blotting and anti-CEA immunoblotting (Fig. 6). This analysis was performed to ascertain whether the major PHA-L reactivity found in these membranes co-localized with CEA immunoreactivity. All tumor samples were shown to have an activating mutation in K-*ras.* First, consistent with previous data (18), all three samples of normal colonic mucosa exhibited minimal PHA-L reactivity. Second, in contrast to normal colonic mucosa, all three tumor samples demonstrated significant PHA-L reactivity at 180 kDa (Fig. 6, left) and CEA immunoreactivity (Fig. 6, right) that co-localized. Comparing the amount of PHA-L reactivity with the amount of CEA immunoreactivity in the three tumor samples revealed that the amount of PHA-L associated with CEA varied between tumor samples. Whether this variation was a reflection of the level of Ras-activation present in these tumors remains to be determined (see Discussion). In summary, the data indicated that the major PHA-L-reactive glycoprotein in primary colon carcinoma was CEA.

DISCUSSION

CEA is a member of the immunoglobulin superfamily and a putative homotypic cell adhesion molecule (19). It is overexpressed in many cancers, including colon carcinoma. CEA has a protein core of 72 kDa, but because of N-linked glycosylation [there are 28 potential N-linked glycosylation sites (20)], it has an apparent molecular weight of approximately 180 kDa. Due to the high number of N-linked glycosylation sites and propensity for overexpression, CEA is an ideal marker to study changes in glycosylation in N-linked carbohydrate in the context of human cancer. In this report we showed that the major PHA-L reactive species in cell lines and primary colonic tumors was CEA, and correlated the amount of β1–6 branching on CEA (the PHA-L/CEA ratio) with Ras activation.

Although the question of whether the addition of β1-6 branching on CEA can modulate CEA function is not known, there is evidence suggesting that the addition of bulky carbohydrate moieties such as polylactosamine to N-linked carbohydrate can interfere with protein–protein interactions. For example, the presence of polylactosamine on fibronectin has shown to inhibit its ability to interact with gelatin (21) and polylactosamine on LAMP-1 has been shown to reduce

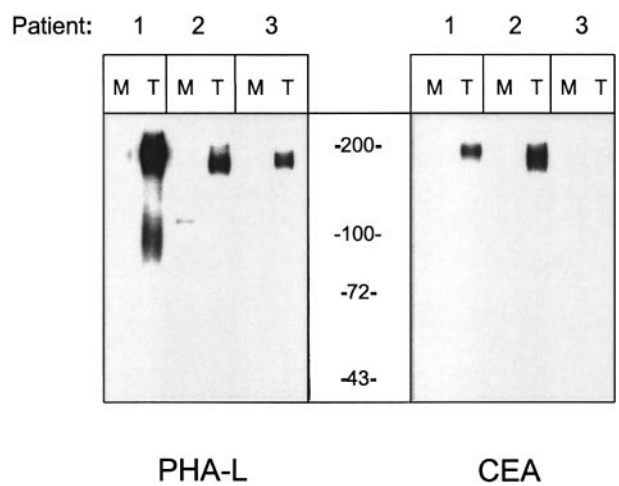

FIG. 6. Detection of PHA-L reactivity and CEA immunoreactivity in cellular membranes derived from primary colon carcinoma. Crude cellular membranes were prepared from tumor (T) and adjacent normal mucosa (M) from three patients with primary colon carcinoma. For each sample, 2.5 μg of membrane protein was separated by SDS–PAGE, transferred to nitrocellulose, and probed with either PHA-L (left) or anti-CEA (right). Bound probe detected as described in the legend to Fig. 1. Center, locations of molecular weight markers (in kDa).

its ability to interact with various extracellular matrix factors (22). The LAMP family and has been shown to be a major substrate for the addition of β1–6 branching in transformed cells and in cells expressing high levels of β1–6 *N*-acetylglucosaminyltransferase V activity (23, 24). The observation that polylactosamine can inhibiting protein-protein interactions is important for a major factor controlling polylactosamine addition to N-linked carbohydrate is β1–6 branching (10). If addition of bulky carbohydrate structures interfere with protein function, then shortening the length of N-linked carbohydrate may have the reverse effect by enhancing protein–protein interactions. An example of this may have been seen when CEA was expressed in a series of CHO cell mutants deficient at different points in the N-linked glycosylation pathway (26). In this study two of three glycosylation deficient mutants were found to exhibit greater CEA-mediated cell–cell adhesion than wild type cells expressing fully glycosylated CEA.

Numerous cell models have shown a relationship between CEA expression and metastatic potential (27, 28). β1–6 branching of N-linked carbohydrate has also been associated with the metastatic phenotype (2). By lectin histochemistry, advanced staged colon carcinomas possess more PHA-L reactivity than early stage colon carcinomas (18, 29) and increased PHA-L reactivity in these tumors has correlated with shorter patient survival (29). We show here that the amount of PHA-L on CEA in the membranes of cell lines, and primary tumors derived from colon carcinoma, contributes significantly to the overall amount of PHA-L reactivity seen in these samples. It would be interesting to determine how closely PHA-L reactivity and CEA immunoreactivity of tumors by histochemistry compare with lectin and immunoblotting of identical tumor samples. Just as important is whether a correlation exists between the PHA-L/CEA ratio and either tumor stage or patient survival; a question not answerable by the use of classic histochemistry.

It has been shown that activated H-*ras* induces higher levels of β1–6 branching than wild-type H-*ras* in transfected fibroblast (8). We have shown that the amount of β1–6 branching correlates with Ras activation in colon carcinoma cell lines (15) and that PHA-L reactivity in PHA-L-resistant cell populations of colon carcinoma cell line HTB39 correlates with the amount of K-Ras protein detected in the membranes of these cells (17). These data, together with the data presented here showing a relationship between the PHA-L/CEA ratio and Ras-activation further supports the viewpoint that Ras-activation can drive the production of β1–6 branching in a quantitative activation-dependent manner. Our studies differ from others in that we show evidence that this may occur within the context of naturally transformed cells. These data also support our hypothesis that the Ras-signaling pathway can be thought of as a continuum of activation, where cellular responses depend upon the level of Ras activation (15).

It has been shown that activated K-Ras, but not activated H-Ras, can induce CEA expression in cells derived from colon carcinoma (30). Thus both β1–6 branching and CEA have been shown to be inducible by K-Ras activation in cell culture. This would suggest that a biochemical analysis of tumors, knowing the K-*ras* genotype, could yield important data correlating the presence of these markers to K-Ras activation *in vivo*. However, cytokine(s) and growth factor(s) alone or in combination have been shown capable of modulating the level of both these markers in transformed cells (31–33). It is possible that elevated levels of such factors in the microenvironment of a tumor may induce/enhance expression of CEA as well as increase the levels of β1–6 branching in the absence of K-*ras* mutation.

Clearly, more work needs to be done to determine whether the PHA-L/CEA ratio can be used as either a marker of Ras activation or as a clinical marker of tumor progression in colorectal carcinoma. In addition, whether β1–6 branching or polylactosamine addition is capable of altering the cell adhesion function of CEA function needs to be explored. Finding that β1–6 branching and by extension polylactosamine does perturb CEA-mediated cell adhesion would provide a mechanistic rationale why the PHA-L/CEA ratio could be an important marker in both a biological and clinical context.

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