Characterization of the Colorectal Carcinoma-associated Antigen Defined by Monoclonal Antibody D612

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ABSTRACT

Monoclonal antibody (MAb) D612 recognizes an antigen expressed on the cell surface of normal and malignant gastrointestinal epithelium. It is a murine IgG2a/k which has been previously shown to mediate killing of human colon carcinoma cells using human effector cells (which could be enhanced in the presence of interleukin-2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of MAb D612 immunoprecipitates of extracts of L-[35S]methionine-, L-[3H]leucine-, and D-[3H]glucosamine-labeled human colon carcinoma cells showed that the D612 antigen is a M, 48,000 glycoprotein. Similar estimates of molecular mass were obtained from SDS-PAGE analyses of MAb D612 immunoprecipitates of radiiodinated extracts of surgically resected colon carcinoma and adjacent normal colonic mucosa. D612 antigen was not detectable in immunoprecipitates of supernatant media from radiolabeled cell cultures, suggesting that the antigen is not readily shed from the surface of cultured cells. The D612 antigen was shown to be clearly distinct from previously described gastrointestinal carcinoma-associated glycoproteins: the D612 antigen shows a migration pattern of SDS-PAGE distinct from those of the antigens recognized by MAb KS1/4 and GA733, and reciprocal immunodepletion analyses of D-[3H]glucosamine-labeled colon carcinoma cells utilizing MAb D612 and GA733 revealed no cross-reactivity between these antibodies. Similarly, competitive binding studies between MAb 17-1A and KS1/4 and MAb D612 revealed no similarity between the epitopes recognized by MAb D612 and MAbs 17-1A and KS1/4. MAbs D612 and 17-1A were also titrated in immunoperoxidase staining assays on serial frozen sections of normal and malignant colon. MAb D612 showed a higher titer of immunostaining reactivity with both normal and malignant colon than did MAb 17-1A. MAb D612 showed roughly equivalent immunostaining titers against normal and malignant colon; whereas MAb 17-1A showed a higher titer of immunostaining reactivity against the normal colon tissue than against the malignant colon. Flow cytometric analysis of phosphorylidyinositol-specific phospholipase C-treated colon carcinoma cells revealed no loss of D612 antigen from the cell surface, suggesting that the mechanism of attachment of the D612 antigen to the cell surface does not involve linkage to a phosphorylidyinositol glycan. Radiolodination of the D612 antigen in a plasma membrane-enriched cell fraction by the photoactivatable carbene-generating reagent, 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine, suggests that the D612 antigen polyepptide penetrates the lipid bilayer of the plasma membrane. It has been determined by Scatchard analysis that the number of binding sites for MAb D612 on the LS-174T human colorectal carcinoma cell line is 4.8 × 10^5. MAb D612 was found to have a Kd of approximately 1.3 × 10^-10 M^-1.

INTRODUCTION

Monoclonal antibodies directed against determinants preferentially associated with malignant gastrointestinal epithelium have found wide ranging application in both the experimental study of normal gastrointestinal cell biology and neoplastic transformation and in the clinical management of gastrointestinal malignancies, including radioimaging of primary and metastatic lesions in situ, differential diagnosis of carcinoma cells in histological or cytological preparations, detection of shed antigens in clinical specimens, and, potentially, immunotherapeutic applications utilizing toxin, drug, or radionuclide-conjugated antibodies or in combination with effector cells. Many of the monoclonal antibodies that have potential or demonstrated utility in the clinical management of gastrointestinal malignancies are directed against determinants expressed on four different families of glycoconjugates: CEA1 and related glycoproteins (1-14), mucins (15-19), glycolipids (20-22), or an M, 35,000-43,000 series of related glycoproteins (23-29).

Recently, Muraro et al. (30) described a monoclonal antibody, D612, that defines an antigen that is expressed on the cell surface of normal and malignant gastrointestinal epithelium and has properties distinct from those of previously described gastrointestinal carcinoma-associated antigens. Immunohistochemical studies have shown that MAb D612 reacts with approximately 85% of primary and approximately 71% of metastatic colorectal carcinomas and with normal gastrointestinal epithelium but does not bind to normal or neoplastic tissue from a wide range of other sites (30). In many cases there was virtually homogeneous staining of the colorectal tumors. In other studies, MAb D612 has shown potential as an immunocytochemical adjunct for the diagnosis of colorectal carcinoma (31). In a study of 62 effusions and fine needle aspirates, MAb D612 reactivity correlated with the correct diagnosis in 92.8% of the instances and clearly distinguished metastatic colon carcinoma from metastatic ovarian, breast, and lung carcinomas (31).

Other studies have suggested that MAb D612 should be considered for potential utility in the immunotherapy of colorectal carcinoma. MAb D612 also mediates a 20- to 30-fold increase in the ADCC of normal human PBMC against human colon carcinoma cells (30, 32). Incubation of the PBMC with 10-500 units/ml of recombinant IL-2 resulted in an additional 2- to 5-fold augmentation of the cytotoxicity of the human effector cells exposed to MAb D612, and the threshold dose of MAb D612 required for efficient ADCC was reduced 200-fold with IL-2 (32). MAb D612 mediated ADCC most actively with nonadherent effector cells, and depletion of FcRγIII-positive lymphoid cells markedly reduced MAb D612-mediated ADCC, demonstrating the participation of natural killer/LAK cells in...
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MAb D612-mediated ADCC (32). Radiolabeled D612 IgG has been shown to selectively localize to a human colon carcinoma xenograft in situ (30). Treatment of athymic mice bearing xenografted human colon carcinomas with uncomplexed MAb D612 resulted in significant inhibition of tumor growth (33). Mouse macrophages, but not splenocytes, mediated ADCC with MAb D612, suggesting that tumor inhibition was mediated by MAb D612 and host macrophages (33). Administration of human LAK cells, generated by incubation of normal human PBMC with 100 units/ml of IL-2 for 24 h, did not significantly inhibit the growth of the xenografted human colon carcinoma (33). However, adoptive transfer of human LAK cells into athymic mice treated with MAb D612 resulted in a significant inhibition of tumor growth, as compared to that obtained with either MAb D612 or LAK cells alone (33). These results demonstrate a synergy between the tumoricidal properties of LAK cells and MAb D612 and suggest a potential immunotherapeutic role for D612 in the management of colorectal carcinoma, possibly as an adjuvant to LAK cell therapy.

In this study, we provide characterization of the antigen defined by MAb D612 and demonstrate that its immunochromical properties are distinct from those of previously described gastrointestinal carcinoma-associated antigens.

MATERIALS AND METHODS

Monoclonal Antibodies and Cell Lines. Monoclonal antibodies D612 and COL-1 were generated as described by Muraro et al. (2, 30). W6/32 (34) was obtained from the American Type Culture Collection (Rockville, MD). MAb D612, COL-1, and W6/32 were purified from murine ascitic fluids by precipitation in 50% saturated ammonium sulfate followed by affinity chromatography on protein A-Atto-Prep (35) (Bio-Rad Laboratories, Richmond CA). Purified MAb 17-1A (36), GA733 (26), and KSI/4 (23) were generously supplied by Dr. Peter Daddona (Centocor, Malvern, PA), Dr. M. Herlyn (Wistar Institute, Philadelphia, PA), and Dr. Leslie Walker (Cytel, La Jolla, CA), respectively. IgG2a/s murine myeloma proteins UPC-10 (37) and RPC5.4 (38) were purchased from Organon Teknika (Durham, NC).

The human colon carcinoma-derived cell line LS-174T (39) and the human melanoma-derived cell line A-375 (40) were obtained from the American Type Culture Collection. The human colon carcinoma-derived cell lines GEO and CBS (41) were obtained from Dr. Michael G. Brattain (Baylor University, Houston, TX). Cell lines derived from solid tumors were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FCS, 2 mM glutamine, Eagle’s minimal essential medium nonessential amino acids, 1 mM sodium pyruvate, and 25 μg/ml gentamicin.

Radiolabeling. For intrinsic radiolabeling, monolayer cells, approximately 50% confluent in 75-cm² flasks, were washed twice with sterile PBS and incubated overnight in 20 ml of sodium-free or leucine-free RPMI 1640 medium (GIBCO) containing 10% FCS, 2 mM glutamine, 25 μg/ml gentamicin, and 2 mM L-15S]cysteine or L-153H]leucine, respectively (Amerham, Arlington Heights, IL) or in 20 ml RPMI 1640 medium (GIBCO) containing 10% FCS, 2 mM glutamine, 25 μg/ml gentamicin, 50 μg/ml glucose, 10 mM sodium pyruvate, and 1 mM D-[1H]glucosamine (Amerham). The labeled cells were then extracted with the nonionic detergent, Renex-30, as described previously (42). Briefly, labeled cells were washed twice with cold PBS and extracted for 20 min on ice in 0.15 M NaCl containing 50 mM Tris, pH 8.5-0.02% sodium azide-5 mM EDTA-2 mM PMSF-2% Renex-30. Following centrifugations at 15,000 × g for 20 min at 4°C and at 100,000 × g for 1 h at 4°C, the radiolabeled supernatant extracts were stored at −70°C for later use. Supernatant medium from the radiolabeled cell cultures was lyophilized, dialyzed extensively against PBS, centrifuged at 100,000 × g for 1 h, and stored at −70°C for later use.

For radiolabeling of cell and tissue extracts, GEO cells and minced and briefly homogenized normal colonic mucosa and colon carcinoma tissue were extracted for 20 min on ice in buffer containing 50 mM sodium borate, pH 8.5-5 mM EDTA-2 mM PMSF-2% Renex-30. Following centrifugations at 15,000 × g for 20 min at 4°C and at 100,000 × g for 1 h at 4°C, human plasma IgG was cleared from the tissue extracts by absorption with protein A-Sepharose conjugate (Phar- macia, Uppsala, Sweden) for 30 min at 4°C. Aliquots of the supernatant extracts containing 100 μg of protein, as determined by a biinchoninic acid assay (43) (Pierce Chemical Co., Rockfall IL), were radioiodinated by the method of Bolton and Hunter (44).

For radioiodination of hydrophilic transmembrane regions of cell surface antigens, a plasma membrane-enriched fraction was prepared from GEO cells as follows. Cells were disrupted on ice with 12 strokes in a ground glass homogenizer in 20 ml of buffer containing 0.15 M NaCl-50 mM sodium borate, pH 8.5-2 mM PMSF; 10 ml of buffer containing 10 mm sodium borate, pH 8.5-1 M EDTA was added; and the preparation was subjected to N2 cavitation at 1000 psi and centrifuged at 1000 × g for 5 min at 4°C. The volume of the supernatant was brought to 72 ml with buffer containing 10 mm sodium borate, pH 8.5-1 mM EDTA and the supernatant was layered over discontinuous 20-40% sucrose gradients in buffer containing 10 mm sodium borate, pH 8.5-1 M EDTA and centrifuged at 100,000 × g for 2 h at 4°C. Material from the interface of the 20-40% sucrose containing 4.2 mg protein, as determined by a biinchoninic acid assay (43) (Pierce), was equilibrated with 250 μCi of the reactive carbene-generating reagent, [125I]TI1D (45, 46) (Amersham) for 1 h on ice in the dark. The preparation was then photolysed at 365 nm for 2 h on ice and centrifuged at 100,000 × g for 1 h at 4°C. Material from the pellet was solubilized in buffer containing 0.15 M NaCl-50 mm Tris, pH 8.5-5 M EDTA-2 mM PMSF-2% Renex-30 and clarified at 100,000 × g for 1 h at 4°C. Five mg/ml of ovalbumin was added, and the radioiodinated preparation was stored at −70°C for future use.

Indirect Immunoprecipitation, Immunodepletion, and SDS-PAGE. Indirect immunoprecipitations were carried out as described previously (42). Briefly, 1-ml aliquots of hybridoma supernatants or 15-30 μg aliquots of purified antibodies in IP buffer were incubated for 1 h at 4°C with 100 μl of 1 M Tris, pH 8.5 and 100 μl of a 10% suspension of protein A-Sepharose conjugate (Phar-macia) in IP buffer. Following two washes with IP buffer, the antibody-coated protein A-Sepharose was incubated for 1 additional h at 4°C with 0.5 ml of IP buffer and 1 × 10⁷ cpm of radiolabeled cell extract. The Sepharose beads were then washed with IP buffer until the cpm eluted in the supernatant buffer were low and stable and then washed twice with IP buffer without ovalbumin.

For immunodepletion analyses, 100-μl aliquots of a 10% suspension of protein A-Sepharose were incubated with 30-μg aliquots of purified D612, GA733, or RPC5.4 in 1 ml of IP buffer for 1 h at 4°C and washed twice with IP buffer. Extracts of radiolabeled GEO cells containing 2 × 10⁷ cpm in 0.5 ml IP buffer were immuno-depleted by three sequential 30-min incubations at 4°C with aliquots of the protein A-Sepharose-bound MAbs. The Sepharose beads were then washed with IP buffer until the cpm eluted in the supernatant buffer were low and stable and then washed twice with IP buffer without ovalbumin. The immunodepleted extracts were subjected to immunoprecipitation with MAb D612 and GA733 as described above. Bound antigens were eluted and separated by SDS-PAGE as described by Laemmli (47). Antigens were visualized by autoradiography as described by Bonner and Laskey (48).

Competition Enzyme Immunoassay. Purified MAbs were conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) by a low pH method of periodate oxidation (49). The wells of flat-bottom 96-well polystyrene microtiter plates were treated with 5 μg of an extract prepared from LS-174T human colon tumor xenografts (50) in 50 μl of PBS. After drying overnight at 37°C, the wells were blocked for 1 h at 37°C with 200 μl of 5% BSA in PBS and then washed three times with 1% BSA/PBS. Varying amounts of unlabeled MAbs in 25 μl of 1% BSA/PBS were added to each well and the plates were incubated for 6 h at 37°C. Enzyme-conjugated MAb (30, 35, and 65 ng of D612-HRPO, KSI/4-HRPO, and 17-1A-HRPO antibody protein, respectively) in 25 μl of 1% BSA/PBS was added to each well, and the
plates were incubated for 18 h at 4°C. The wells were again washed three times with 1% BSA/PBS and then exposed to substrate solution containing 0.015% H₂O₂ and 2.8 mm α-phenylenediamine (Sigma) in 0.1 M phosphate-citrate buffer, pH 5.0. After 15 min, 25 μl of 4 M H₂SO₄ was added to each well and the absorbance at 490 nm was read.

Immunoperoxidase Staining. Immunoperoxidase staining was carried out essentially as previously described (30).

Phosphatidylinositol Phospholipase C Digestion and Fluorescence Flow Cytometry. GEO cells were trypsinized from subconfluent monolayers and approximately 6 × 10⁶ cells were resuspended in PBS containing 1% BSA and 1.0 unit of PI-PLC (Bacillus cereus; Boehringer Mannheim Biochemicals). One unit is the enzyme activity which hydrolyzes 1 μmol phosphatidylinositol/min at pH 7.5 and 37°C. The cells were incubated at 37°C for 1 h and washed three times with cold Ca²⁺, Mg²⁺-free PBS. The cells were then centrifuged and washed three times with cold Ca²⁺, Mg²⁺-free PBS. The cells were then incubated in a 1:50 dilution of fluorescein isothiocyanate anti-mouse IgG (Cooper Biomedical, Malvern, PA) in 1% BSA/PBS for 1 h at 4°C. The cells were then centrifuged and washed three times with cold Ca²⁺, Mg²⁺-free PBS and analyzed with a Becton-Dickinson FACScan (Mountain View, CA) equipped with blue laser excitation of 15 mW at 488 nm. The results shown in Fig. 8 were generated from the analysis of 10,000 cells collected with a live gate.

Determination of Affinity and Sites/Cell. The affinity of MAb D612 and the maximum number of antibody-binding sites on LS-174T cells were determined utilizing MAb's labeled with ¹²⁵I by the iodogen method (52) (Pierce) to 10 μCi/μg and nitrocellulose membrane-sealed, 96-well millipore plates (Millipore Corp., Bedford, MA) according to the procedures of Mason and Williams (53). For determination of the kᵣ, 50 μl of labeled MAb D612 containing 5 × 10⁵ cpm was added to 1 × 10⁶ LS-174T cells suspended in RPMI 1640 medium + 2% PCS + 0.01 M NaN₃ and incubated with agitation at 4°C. Cell-associated antibody was measured after removal of free MAb by vacuum filtration after various times (2, 4, 6, 8, 10, 12, 15, 30, 60, and 120 min). Maximum MAb binding was determined by incubation at 4°C for 24 h with serial 2-fold dilutions of labeled MAb D612 (5 × 10⁻²-2 × 10⁶ cpm). Nonspecific binding was estimated for incubation of labeled MAb D612 in the presence of a 50-fold excess of unlabeled MAb D612. The association rate constant and the maximum number of binding sites/cell were then calculated by the methods of Mason and Williams (53) and Satchard (54).

RESULTS

Immunoprecipitation Analysis. Immunoprecipitation analyses were utilized to characterize and compare the molecular masses of the D612 antigen derived from human colon carcinoma-derived cell lines, from a surgically resected colon carcinoma, and from the adjacent normal mucosa of the surgically resected colon carcinoma. Fig. 1 shows SDS-PAGE analyses of negative control myeloma protein (lanes A, C, E, G, I, K, M, and O) and MAb D612 (lanes B, D, F, H, J, L, N, and P) immunoprecipitates of nonionic detergent extracts of radiolabeled human cell lines. An M, 48,000 band is precipitated by MAb D612 from extracts of GEO human colorectal carcinoma cells radiolabeled with L-[³⁵S]cysteine (lane B), L-[³⁵S]leucine (lane D), and d-[³⁵S]glucosamine (lane F) and from extracts of CBS human colorectal carcinoma cells radiolabeled with L-[³⁵S]leucine (lane H) and d-[³⁵S]glucosamine (data not shown). Successful radiolabeling of the D612 antigen with both polyepptide and oligosaccharide precursors indicates that the D612 antigen is a glycoprotein. MAb D612 is nonreactive in radioimmunoassays against the human melanoma-derived cell line, A375 (30), and no bands are visualized in SDS-PAGE analyses of D612 immunoprecipitates of nonionic detergent extracts of A375 cells radiolabeled with L-[³⁵S]leucine (lane L) and d-[³⁵S]glucosamine (lane N). Under nonreducing conditions, SDS-PAGE analysis of MAb D612 immunoprecipitates of extracts of L-[³⁵S]cysteine-labeled GEO cells reveals two diffuse bands of slightly greater mobility (lane P).

To confirm the molecular mass of the D612 antigen in vivo in normal and malignant colon, extracts of these tissues, as well as GEO cells, were radioiodinated by the method of Bolton and Hunter (44) and subjected to immunoprecipitation analysis. Fig. 2 shows SDS-PAGE analysis of isotype-matched negative control myeloma protein (lanes A, C, and E) and MAb D612 (lanes B, D, and F) immunoprecipitates of radioiodinated extracts of GEO cells (lanes A and B), normal human colon mucosa (lanes C and D), and a freshly resected human colon carcinoma (lanes E and F). Although the reagent of Bolton and Hunter radioiodinated the D612 antigen with poor efficiency relative to MHC class I antigens and the K51/4 antigen (data not shown), an M, 48,000 band was specifically precipitated by MAb D612 from all three radioiodinated extracts.

Comparison of D612 Antigen with Other Gastrointestinal Carcinoma-associated Antigens. Immunoprecipitation, reciprocal immunodepletion, and competitive binding analyses were carried out to determine whether MAb D612 recognizes an antigen related to previously described gastrointestinal carcinoma-associated glycoprotein antigens. Fig. 3 shows the results of SDS-PAGE analysis of negative control myeloma protein
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Fig. 2. Immunoprecipitates of radioiodinated extracts of GEO human colon carcinoma cells (lanes A and B), normal colonic mucosa (lanes C and D), and colon carcinoma (lanes E and F). Lanes A, C, and E, RPC5.4 myeloma protein (negative control) immunoprecipitates; lanes B, D, and F, MAb D612 immunoprecipitates. *Vertical axis, M, ×1000.

Fig. 3. Immunoprecipitates of extracts of D-[3H]glucosamine-labeled GEO human colon carcinoma cells. Lane A, RPC5.4 myeloma protein (negative control); lane B, MAb D612; lane C, MAb GA733; lane D, MAb KS1/4. Vertical axis, M, ×1000.

RPC5.4 (lane A), MAb D612 (lane B), MAb GA733 (lane C), and MAb KS1/4 (lane D) immunoprecipitates of a nonionic detergent extract of D-[3H]glucosamine-labeled GEO cells. Clearly, the Mₐ 48,000 D612 antigen has a mobility distinct from those of the GA733 and KS1/4 antigens, which show major components of approximately Mₐ 42,000, 40,000, and 35,000.

To rule out any cross-reactivity between these antigens, reciprocal immunodepletion analyses were carried out between MAbs D612 and GA733. Fig. 4 shows the results of SDS-PAGE analysis of MAb D612 (lane D) and MAb GA733 (lane E) immunoprecipitates of a nonionic detergent extract of D-[3H]glucosamine-labeled GEO cells subjected to three rounds of immunodepletion with solid phase MAb D612 (lanes A–C). All detectable D612 antigen was depleted from the extract prior to the third incubation with solid phase MAb D612 (lane C) and no D612 antigen is visualized in lane D, while roughly the same amount of GA733 antigen is immunoprecipitated from the MAb D612-depleted extract (lane E) as from a similar extract subjected to three rounds of immunodepletion with a myeloma protein in solid phase (lane F). Fig. 5 shows the results of the reciprocal experiment: SDS-PAGE analysis of MAb GA733 (lane D) and MAb D612 (lane E) immunoprecipitates of a nonionic detergent extract of D-[3H]glucosamine-labeled GEO cells subjected to three rounds of immunodepletion with solid phase MAb GA733 (lanes A–C). All detectable GA733 antigen was depleted from the extract prior to the third incubation with solid phase MAb GA733 (lane C) and no GA733 antigen is visualized in lane D, while roughly the same amount of D612 antigen is immunoprecipitated from the MAb GA733-depleted extract (lane E) as from a similar extract subjected to three rounds of immunodepletion with a myeloma protein in solid phase (lane F).

Reciprocal cross-competition assays were carried out to determine whether the epitope recognized by MAb D612 has any similarity to those reacting with MAbs KS1/4 or 17–1A. As
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Fig. 6. Reciprocal competition enzyme immunoassay between MAbs D612, KS1/4, and 17-1A. MAbs D612 (•), KS1/4 (D), and 17-1A (○) and UPC-10 myeloma protein (A) were used as competitors against horseradish peroxidase-labeled MAbs D612 (4), KS1/4 (B), or 17-1A (C). In the experiment shown, the A405 minus background in the absence of competitor was 2.1, 1.8, and 1.0 for the enzyme-labeled MAbs D612, KS1/4, and 17-1A, respectively.

shown in Fig. 6A, low amounts of MAb D612 were active in homologous competition against MAb D612-HRPO, while MAbs KS1/4 and 17-1A both failed to compete at antibody levels up to 20 μg/well. MAbs KS1/4 and 17-1A were both active in homologous competition (Fig. 6, B and C, respectively). Reciprocal cross-competition is also evident between MAbs KS1/4 and 17-1A, whereas MAb D612 was completely ineffective in competing against these latter MAbs. MAb KS1/4 was a more efficient competitor than MAb 17-1A in both homologous and heterologous assays using MAbs KS1/4-HRPO (Fig. 6B) or MAb 17-1A-HRPO (Fig. 6C). MAbs D612 and 17-1A were also titrated in immunoperoxidase staining assays on serial frozen sections of normal and malignant colon. As shown in Fig. 7, MAb D612 showed a higher titer of immunostaining reactivity with normal and malignant colon than did MAb 17-1A. More significantly, however, MAb D612 showed roughly equivalent immunostaining titers against normal and malignant colon, whereas MAb 17-1A actually showed a higher titer of immunostaining reactivity against the normal colon tissue than against the malignant colon.

Cell Surface Linkage and Shedding of the D612 Antigen. Anchorage to the cell surface by covalent linkage of the COOH-terminal residue through ethanolamine to a complex PI-G tail has recently been described for more than 20 mature proteins (for review, see Ref. 55), including CEA (56). Evidence for this type of attachment includes the release of the membrane-bound antigen following digestion with PI-PLC. To determine whether the D612 antigen is attached to the cell surface by the PI-G tail linkage, GEO cells were incubated with PI-PLC or a buffer control prior to indirect immunofluorescent staining by flow cytometry. As shown in Fig. 8A, the level of MAb D612 binding to GEO cells is unaffected following treatment with PI-PLC. In contrast, as shown in Fig. 8B, treatment of GEO cells with PI-PLC resulted in the removal of approximately 80% of cell surface CEA, as measured by the binding of MAb COL-1. These results suggest that the D612 antigen is anchored to the membrane by a mechanism other than the PI-G moiety.
To determine whether the D612 antigen polypeptide penetrates the lipid bilayer of the cell membrane, a plasma membrane-enriched fraction prepared from GEO cells was radioiodinated with the photoactivatable, carbene-generating reagent, \([^{125}\text{I}]\text{TID}\), which labels transmembrane domains of intrinsic membrane proteins in a highly selective manner (45, 46), and extracted with a nonionic detergent. Fig. 9 shows the results of SDS-PAGE analysis of negative control (lane A), MAb D612 (lane B), MAb W6/32 (lane C), and MAb KS1/4 (lane D) immunoprecipitates of the \([^{125}\text{I}]\text{TID}\)-labeled membrane extract. The D612 antigen is radioiodinated with very high efficiency by \([^{125}\text{I}]\text{TID}\) relative to the M, 45,000 MHC class I heavy chain precipitated by MAb W6/32 and the KS1/4 antigen, which are also clearly radiolabeled by this method. The M, 12,000 MHC class I light chain, which does not penetrate the lipid bilayer of the plasma membrane, is not visualized in lane C.

To determine whether the D612 antigen is shed from the surface of cultured human colon carcinoma cells into the external medium, supernatant media from radiolabeled human colon carcinoma cell cultures were concentrated and clarified at 100,000 x g to remove membrane fragments and subjected to immunoprecipitation analyses. Fig. 10 shows the results of SDS-PAGE analyses of negative control myeloma protein (lanes A, D, and G), MAb D612 (lanes B, E, and H), and MAb COL-1 (lanes C, F, and I) immunoprecipitates of supernatant media from GEO cells labeled with l-[\(^{35}\text{S}\)]cysteine (lanes A–C) and d-[\(^{3}\text{H}\)]glucosamine (lanes D–F) and from CBS cells radiolabeled with d-[\(^{3}\text{H}\)]glucosamine (lanes G–I). CEA is readily detected in COL-1 immunoprecipitates of the supernatant media from the radiolabeled cells, while D612 antigen is not detectable. Similar results were obtained using supernatant medium from d-[\(^{3}\text{H}\)]glucosamine-labeled LS-174T cells (data not shown), although lower amounts of CEA were detected. These results suggest that the D612 antigen is not shed from the surfaces of cultured cells in detectable amounts.

**Fig. 9.** Immunoprecipitates of extract of GEO cell plasma membrane preparation radioiodinated with 3-[[fluoromethyl]-3-(m-[\(^{125}\text{I}]\text{iodophenyl)diazirine.}](lanes A, D, and G), MAb D612 (lanes B, E, and H), and MAb KS1/4 (lane D) immunoprecipitates of extracts of GEO cell plasma membrane preparation radioiodinated with 3-[[fluoromethyl]-3-(m-[\(^{125}\text{I}]\text{iodophenyl)diazirine.}](lanes A, D, and G), MAb D612 (lanes B, E, and H), and MAb KS1/4 (lane D)

**Fig. 10.** Immunoprecipitates of supernatant media from GEO human colon carcinoma cells radiolabeled with L-[\(^{35}\text{S}\)]cysteine (lanes A–C) or d-[\(^{3}\text{H}\)]glucosamine (lanes D–F) or CBS human carcinoma cells radiolabeled with d-[\(^{3}\text{H}\)]glucosamine (lanes G–I). Lanes A, D, and G, RPC5.4 myeloma protein (negative control); lanes B, E, and H, MAb D612; lanes C, F, and I, MAb COL-1. Vertical axis, M, ×1000.

**DISCUSSION**

The principal observations of this study are as follows: (a) MAb D612 recognizes an M, 48,000 glycoprotein on the cell surface of normal and malignant gastrointestinal epithelium; (b) the D612 antigen appears to be an integral membrane glycoprotein which is not readily shed from the cell surface; and (c) the D612 antigen has immunochemical properties that clearly distinguish it from previously described gastrointestinal carcinoma-associated glycoproteins.

Previously reported estimates of M, >1,000,000 for the molecular mass of the D612 antigen obtained by Western blotting analyses (30) were not correct. The epitope recognized by MAb D612 is highly sensitive to denaturation, and the D612 antigen is not detectable in Western blotting analyses. The high molecular weight band immunostained by Muraro et al. (30) most likely represents some incompletely solubilized or non-denatured material from the extract which, therefore, retained some immunoactivity and failed to migrate into the gel. The results of SDS-PAGE analysis of D612 immunoprecipitates of extracts of L-[\(^{35}\text{S}\)]methionine-, L-[\(^{3}\text{H}\)]leucine-, and d-[\(^{3}\text{H}\)]glucosamine-labeled colon carcinoma cells clearly show that the D612 antigen is an M, 48,000 glycoprotein. Since the D612 antigen cannot be visualized by conventional Western blotting procedures, radiiodination of tissue extracts by the method of Bolton and Hunter (44), followed by immunoprecipitation, was used to confirm the molecular mass of the D612 antigen in normal and malignant colon tissues. Relative to MHC class I antigens and the KS1/4 antigen, the D612 antigen is radioiodinated with poor efficiency by the method of Bolton and Hunter, resulting in significantly higher backgrounds, even with the GEO cell extract, than are obtained by metabolic radiolabelling.
beling and immunoprecipitation. However, $M$, 48,000 bands are visualized in SDS-PAGE analyses of MAb D612 immunoprecipitates of radioiodinated extracts of GEO cells, a surgically resected colon carcinoma, and adjacent normal colonic mucosa.

There is some compression and distortion of the immunoprecipitated D612 antigen band on SDS-PAGE due to the proximity of excess unlabeled D612 IgG heavy chain eluted from the immunoprecipitates. However, under nonreducing SDS-PAGE, the D612 antigen migrates as two separate diffuse bands having slightly increased mobility. It is presently unclear whether the two bands visualized under nonreducing conditions may represent isoforms of the D612 antigen whose existence is obscured under reducing conditions by the IgG heavy chain. The increased mobility of the D612 antigen under nonreducing conditions is consistent with the highly efficient radiolabeling of the antigen by L-${}^{35}$S cysteine and is suggestive of the existence of cystine-stabilized domain structures within the antigen molecule.

Recently, a complex PI-G tail covalently linked through ethanolamine to the COOH-terminal residue has been described for attachment of more than 20 mature proteins to the cell surface (55). Evidence for this type of attachment includes the release of the membrane-bound antigen following digestion with PI-PLC. The PI-PLC cleaves the PI-G-tailed antigens between the phosphate and the 1,2-diacylglycerol moiety resulting in the release of the antigen from the cell surface. However, flow cytometric analysis of PI-PLC-treated colon carcinoma cells revealed no loss of D612 antigen from the cell surface, suggesting that the mechanism of attachment of the D612 antigen to the cell surface does not involve linkage to a phosphatidylinositol glycan. These results are consistent with the successful radioiodination of the D612 antigen in a plasma membrane-enriched cell fraction by the photoactivatable carbene-generating reagent, 3-(trifluoromethyl)-1-(3-iodo-4-phenyl)diazirine. Radioiodination by this method of the MHC class I heavy chain, but not the light chain, confirms the specificity of this method for the selective radiolabeling of transmembrane regions of cell membrane polypeptides. The highly efficient radiolabeling by $[{}^{125}$I]TID of the D612 antigen relative to the MHC class I antigens and the KS1/4 antigen, which possess single transmembrane domains (57, 58), suggests that the D612 antigen may possess multiple transmembrane domains, since the D612 antigen does not seem to be expressed on cell surfaces in greater numbers than the glycoproteins recognized by KS1/4, 17–1A, and related MAbs (23–29). Taken together, these results suggest that the D612 antigen peptide is embedded in the lipid bilayer of the plasma membrane. D612 antigen is not detectable in immunoprecipitates of supernatant media from radiolabeled cell cultures, suggesting that this integral membrane glycoprotein antigen is not readily shed from the surface of cultured cells.

The D612 antigen is clearly distinct from the $M$, 35,000–43,000 series of related carcinoma-associated glycoproteins recognized by MAbs 17–1A, GA733, KS1/4, L-D1, MH99, HEA125, and AUAI (23–29). The number of binding sites/LS-174T cell for MAb D612 was found by Scatchard analysis (54) to be slightly less than one-half the number of binding sites for the less avid MAb 17–1A. The electrophoretic mobility of the D612 antigen is distinct from that of the KS1/4 and GA733 antigens, and reciprocal immunodepletion analyses of $[{}^{3}$H] glucosamine-labeled colon carcinoma cells utilizing MAbs D612 and GA733 revealed no cross-reactivity between these antibodies. Similarly, reciprocal competitive binding studies between MAbs 17–1A and KS1/4 and MAb D612 revealed no similarity between the epitopes recognized by MAb D612 and MAb KS1/4. Low amounts of MAb D612 were active in homologous competition against MAb D612-HRPO, while MAbs KS1/4 and 17–1A both failed to compete at antibody levels up to 20 μg/well. MAbs KS1/4 and 17–1A were both active in homologous competition, and reciprocal cross-competition was also evident between these MAbs, although MAb KS1/4 was a more efficient competitor than MAb 17–1A in both homologous and heterologous assays using MAb KS1/4 HRPO or MAb 17–1A HRPO, respectively. MAb D612 was completely ineffective in competing against MAb KS1/4 and 17–1A. MAb D612 and 17–1A were also titrated in immunoperoxidase staining assays on serial frozen sections of normal and malignant colon. MAb D612 showed a higher titer of
immunostaining reactivity with normal and malignant colon that did MAb 17–1A. More significantly, MAb 17–1A showed a higher titer of immunostaining reactivity against the normal colon tissue than against the malignant colon, while MAb D612 showed roughly equivalent immunostaining titers against normal and malignant colon.

The studies reported here thus define and characterize the M, 48,000 glycoprotein recognized by MAb D612. This antigen may be useful in the identification and characterization of cells of gastrointestinal origin and may prove useful as a target for MAb-based diagnosis or perhaps therapy.

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Characterization of the Colorectal Carcinoma-associated Antigen Defined by Monoclonal Antibody D612

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