Integrin α5/β1 Expression Mediates HER-2 Down-regulation in Colon Cancer Cells*

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HER-2 is constitutively activated and overexpressed in many cancers, and its inhibition in colon cancer cells diminishes tumorigenicity and induces apoptosis. Little is known about the regulation of HER-2 signaling in colon cancer cells. Integrin α5/β1 expression is frequently lost in colorectal cancer cells compared with normal intestinal epithelium, and colon cancer cells lacking integrin α5/β1 expression utilize HER-2 signaling for proliferation and tumorigenicity. Re-expression of integrin α5/β1 in colon cancer cells abrogated their tumorigenicity, but how this occurs is not well known. Stable expression of integrin α5/β1 in colon cancer cells with little or no detectable integrin α5/β1 protein expression resulted in the post-transcriptional down-regulation of HER-2 protein. Integrin α5/β1 was found to interact with HER-2, and the cytoplasmic domain of integrin α5/β1 was sufficient to mediate HER-2 down-regulation. Integrin α5/β1-mediated down-regulation of HER-2 was the result of increased lysosomal targeting. The inhibition of HER-2 signaling represents a potential mechanism by which integrin α5/β1 exerts its tumor suppressor-like activity in colon cancer cells. These results also suggest that a novel function for integrin α5/β1 is the control of HER-2 expression.

The EGFR1 is commonly overexpressed in colorectal cancers and has successfully been targeted in a recently completed human clinical trial (1). HER-2 (human epidermal growth factor receptor-2), a member of the c-erbB receptor tyrosine kinase protooncogene family, is commonly overexpressed in a variety of cancers, including 35–85% of human colorectal cancers, in which its overexpression is associated with a poor prognosis (2–4). However, its potential role as a therapeutic target in colon cancers is in the preclinical phase. HER-2 is the only HER family member that lacks a ligand, but its tyrosine kinase activity can be stimulated through heterodimerization with other activated HER family members. It appears to be the preferred heterodimerization partner when co-expressed with other HER family members and promotes cancer cell survival, proliferation, and metastasis (5). HER-2 can also stimulate the expression of vascular endothelial growth factor and cyclooxygenase-2 by cancer cells, thus promoting tumor angiogenesis and survival (6, 7). HER-2 is an important therapeutic target in breast cancers, especially in those that greatly overexpress HER-2 protein. Colon cancer cells express much less HER-2 relative to breast cancer cells, but preclinical studies suggest that HER-2 may still be a relevant therapeutic target in human colon cancers (8, 9). Inhibition of HER-2 in colon cancer cells lacking detectable integrin α5/β1 expression resulted in diminished cell proliferation and tumor formation in athymic mice (9). c-erbB-2 gene duplication appears to be an uncommon cause of HER-2 protein overexpression in colorectal cancers (10), and the post-translational regulation of HER-2 in colorectal cancers is poorly understood.

Integrins are the largest family of epithelial cell adhesion receptors and are expressed in cells as α/β heterodimers. Integrins and the HER family share common signaling pathways and cellular responses, such as differentiation, survival, motility, and proliferation. In fact, integrins can activate the EGFR in epithelial and nonepithelial cell lines (11–13). Regulation of integrin and HER family members is critical to normal cell behavior, because the dysregulation of the expression and function of members of each of these receptor families promotes tumorigenicity (14, 15). Integrin α5/β1 expression is commonly lost during colonic tumorigenesis, and its re-expression in malignant Chinese hamster ovary and colon cancer cells abrogated their anchorage independent growth and tumorigenicity (13, 16–18). Little is known to the mechanisms of the tumor suppressor-like activity of integrin α5/β1.

We show that the stable expression of integrin α5/β1 in colon cancer cells lacking integrin α5/β1 expression caused a significant down-regulation of HER-2 protein levels. The down-regulation of HER-2 was not due to changes in c-erbB-2 transcriptional regulation. In addition, stable expression of integrin α5/β1 in a colon cancer cell line resulted in a significant decrease in amphiregulin mRNA levels and the loss of constitutive EGFR autophosphorylation, which was seen in the control cells. These results suggest that the loss of integrin α5/β1 expression in colon cells may lead to constitutive activation of EGFR and HER-2-mediated signaling, which in turn promotes growth factor and extracellular matrix independence.

EXPERIMENTAL PROCEDURES

Reagents and Constructs

Humanized mAb 4D5 (Herceptin™) was purchased from the Huntsman Cancer Institute pharmacy. The following antibodies were used for the immunoblotting experiments: integrin α5 mAb (BD Transduction Laboratories), HER-2 mAb (Ab-3, Oncogene Research), phospho-MAPK mAb (Cell Signaling), ERK1 mAb (Transduction Laboratories), actin mAb (Zymed Laboratories Inc.), V5 mAb (Invitrogen), Myc polyclonal antibody (Roche Applied Science), and ubiquitin polyclonal antibody (Santa Cruz Biotechnology). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse antibodies (Zymed Laboratories Inc.). Lysosomes were detected using the acidophilic and fluorescent Lysotracker® dye (Molecular Probes). The lysosomal inhibitor chloroquine (Sigma) was used at a final concentration of 4 μg/ml in

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The abbreviations used are: EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; ERK, extracellular signal-regulated kinase; eGFP, enhanced green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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the cell culture medium. pcDNA3-integrin α5 was previously con-structed as published (13).

pTP2000-HER-2-eGFP

Site-directed mutagenesis of pTP2000-HER-2 (a kind gift from Dr. Kathleen Woods Ignatowski, University of Michigan) was performed to generate a Sall site before the stop codon after the HER-2 sequence. A DNA fragment encoding eGFP with Sall restriction sites at the 5‘ and 3‘ ends was generated by PCR of the vector eGFP (Amaxa) using the following primers: 5‘-TACCGGGTCGACCATGTTGGAG- CAAGGCCGGA-3‘ (sense primer) and 5‘-GATCCGGTCGACCTGCA- GGGTGGACA (antisense primer). The cDNA was designed to con-template with the following primers: CCAAGAATTCAGGCAGCTATG- GGATCCTGGAACCTGGAACTCACCTACCT-3‘ (antisense primer). The PCR fragment was designed to contain 5‘ BamHI and SmaI sites at the 5‘ ends, respectively, using the

pcDNA4-Δcyto

pcDNA3α5 was cut with the restriction endonuclease HindIII. The truncated integrin α5 DNA sequence was gel-purified and subcloned into the HindIII site of pcDNA4B (Invitrogen). The resulting construct encoded a peptide in which the cytoplasmic tail of the integrin α5 subunit was deleted at the amino acid just after the transmembrane sequence: extracellular domain–transmembrane sequence–KL/GFFK- RSLPYGTAMEKAQLKPPATSDA (the cytoplasmic domain is under-lined, and the forward slash shows the location of the truncation).

pcDNA4-SECD

First, a plasmid containing the integrin α5 signal sequence was made. The integrin α5 signal sequence (MGSRTTPESPLHAVQLRGW- RRPRFLPLLFLLPPLPPRPGFVN) was amplified by PCR using pcDNA3-α5 as a template and with the following primers: 5‘- CGCTAAGCCTAGGAGGGGCTTATGGAGGCCC-3‘ (sense primer) and 5‘-CTGGGATCCCTGCCCGTCTAATGTTAAGGCC-3‘ (antisense primer). The PCR fragment was designed to contain 5‘ HindIII and 3‘ BamHI restriction sites. The fragment was cut with HindIII and BamHI, gel-purified, and ligated in frame into pcDNA4α-MycC-V5-HisE. The resulting pcDNA4-α5 SS (signal sequence)+MycC-V5-HisE construct was sequence-verified. The integrin α5 sequence with deletion of the extracellular domain was constructed next. A DNA fragment containing the transmembrane and cytoplasmic domains was generated by PCR using pcDNA3-α5 as a template with the following primers: CCAGAAATTCGAAGCGGCTATG- GGTCCTACCTGT (sense primer) and AAATCTCGAGGACTCGG- GCATCAGGTT (antisense primer). The cDNA was designed to con-tain EcoRI and XhoI restriction sites at the 5‘ and 3‘ ends, respectively. The PCR product was cut with EcoRI and XhoI, gel-purified, and ligated in frame into pcDNA4-α5 SS-MycC-V5-HisE, which had been digested with EcoRI and XhoI (between the MycC and V5 epitope tag sequences). This re-sulted in a truncation mutant encoding an integrin α5 protein missing the extracellular domain–GSGYGVPLWHIALFGLLGLLLILYLILYL–cyto-plasmic domain (the transmembrane domain is underlined, and the forward slash shows the location of the 5‘ end of the truncation).

pcDNA4-Tm

The integrin α5 transmembrane sequence (GSYGVPVLWHIALF- GLLGLLLILYLIL) was amplified by PCR using pcDNA3-α5 as a template and with the following primers: 5‘-CCAGAAATTCGAGC- GCTATGCGGCTACCTG-3‘ (sense primer) and 5‘-TTTGAATCTCG- AAGAGCTTTAGAGATGAGATGAG-3‘ (antisense primer). The cDNA was designed to contain EcoRI and XhoI restriction sites at the 5‘ and 3‘ ends, respectively. The PCR product was cut with EcoRI and XhoI, gel-purified, and ligated in frame into pcDNA4-α5 SS-MycC-V5-HisE, which had been digested with EcoRI and XhoI (between the MycC and V5 epitope tag sequences). The final construct was sequence-verified.

Cell Lines and Culture

Caco-2 and HT-29 colon cancer cell lines were obtained from the ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, streptomycin, and gentamycin. The cells were cultured to about 70% confluency on plain dishes, and Caco-2 cells were cultured to about 70% confluency on plain dishes. The medium was removed, and the cells were washed with 4 ml of OptiMEM medium (Invitrogen) and then overlaid with 2.4 ml of OptiMEM medium/dish. A mixture of 250 μl of OptiMEM containing 4 μg of DNA that was pre-mixed with 8 μl of Plus reagent (Invitrogen) was incubated at room temperature for 15 min. The DNA mixture was then added to 250 μl of OptiMEM plus 24 μl of Lipofectamine (Invitrogen), which was then incubated at room temperature for 15 min. The DNA mixture was added to the cells, which were then incubated in the tissue culture incubator for 3 h. The medium was removed, and the cells were overlaid with complete DMEM for transient transfections or complete DMEM with 1.5 mg/ml G418 (pcDNA3-based constructs) or 200 μg/ml zeocin (for pcDNA4-based constructs).

For transient expression of pcDNA4-HER2-eGFP in HT-29 cells, the NucleofectorTM (Amaxa) was used because these cells were difficult to transfect. Confluent monolayers of HT-29 cells in 60-mm dishes were dispensed in trypsin-PBS, and then 5 ml of medium was added to each dish of cells. The cells were plated in conical sterile tubes and centrifuged at 1,000 rpm for 3 min at room temperature. The medium was removed, and the cells were washed twice in PBS. The cells were resuspended in 100 μl of Nucleofector Solution V (Amaxa) and then mixed with 5 μl of DNA (concentration, 1 μg/μl). The cell/DNA mixture was transferred to a cuvette (Amaxa) and placed in the NucleofectorTM device. The samples were electroprogrammed using program T-20. The cuvettes were immediately removed, and 500 μl of RPMI (supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glucose) was added to the cells. The cells were then transferred to 25-mm dishes containing sterile coverslips immersed in DMEM with 75% DMEM (final concentration) Lysotracker® dye. At various time points, the cells on the coverslips were washed with PBS and fixed in 4% paraformalde-hyde in PBS for 10 min. The cells were washed twice in PBS and then mounted in Prolong Gold® (Molecular Probes) on glass slides. The cells were imaged at 100× on a fluorescent microscope.

Soft Agar Proliferation Assay

A 1.6% low melting point agar solution (in water) was microwaved until boiling and then cooled in a 37 °C water bath. The agar solution was mixed 1:1 with 2× medium, and 1 ml of this solution was plated in 35-mm tissue culture plastic dishes. 20,000 cells were added to a solution containing 2× medium and 1× medium in a 1:2 ratio (3 ml total volume/dish). Then 1 ml of liquid 1.6% agar solution (at 37 °C) was added to the cell mixture, which was vortexed gently to disperse the cells. One ml of the cell mixture was layered onto the bottom layer and left at room temperature until the agar solidified. The cells were placed in a 37 °C incubator with 5% CO2 and photographed each day.

Northern Blot

The following probes were constructed. GAPDH—A 1272-base pair DNA fragment was cut out of pBS/KS II-GAPDH plasmid (which contains the full length cDNA) gel-purified and gel-purified. HER2—A 210-base pair PCR product was generated from Caco-2 cDNA that encompassed the exon II–III splice site and incorporated BamHI and Smal sites at the 5‘ and 3‘ ends, respectively, using the following set of primers: forward primer with BamHI site, 5‘-GAGT- GGATCCGGAACTGGAACCTACTCACCTC-3‘, and reverse primer with Smal site, 5‘-AAATACCGGGTGTATGTGCAGC-3‘. The PCR product was cut with the restriction endonucleases BamHI and Smal, which were maintained on fibronectin-coated plastic in medium supplemented with 1 mg/ml of G418 (17).

Transfection Protocols

HEK 293 cells were cultured in DMEM complete medium to about 70% confluency on poly-l-lysine-coated dishes, and Caco-2 cells were cultured to about 70% confluency on plain dishes. The medium was removed, and the cells were washed with 4 ml of OptiMEM medium (Invitrogen) and then overlaid with 2.4 ml of OptiMEM medium/dish. A mixture of 250 μl of OptiMEM containing 4 μg of DNA that was pre-mixed with 8 μl of Plus reagent (Invitrogen) was incubated at room temperature for 15 min. The DNA mixture was then added to 250 μl of OptiMEM plus 24 μl of Lipofectamine (Invitrogen), which was then incubated at room temperature for 15 min. The DNA mixture was added to the cells, which were then incubated in the tissue culture incubator for 3 h. The medium was removed, and the cells were overlaid with complete DMEM for transient transfections or complete DMEM with 1.5 mg/ml G418 (pcDNA3-based constructs) or 200 μg/ml zeocin (for pcDNA4-based constructs).
gel-purified, and ligated into the BamHI and EcoRV sites of pcDNA4C (Invitrogen). The probe for Northern blotting was cut out with BamHI and NotI and gel-purified. The probes were randomly labeled with [32P]dCTP using the RapidPrime DNA labeling system (Invitrogen) and purified with G-50 Sephadex quick spin columns (Roche Applied Science).

RNA was extracted from 80–90% confluent cell monolayers with the RNeasy kit (Qiagen) per the manufacturer’s instructions. The RNA was resolved in formaldehyde, 0.9% agarose gels and transferred to Hybond-N+ Nylon membranes (Amersham Biosciences) and immobilized with UV light. The membranes were washed twice in prehybridization buffer (5× Denhardt’s Solution, 5× SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, 50% formamide, and 250 μg/ml denatured salmon sperm single-stranded DNA at 42 °C for 3 h. The denatured probe was then added into the buffer and incubated at 42 °C overnight. The membranes were washed in 2× SSC with 0.1% SDS at room temperature for 5 min and then twice for 20 min each in 0.1× SSC with 0.1% SDS at 65 °C. X-ray film was exposed to the membranes with intensifying screens at −70 °C overnight and then developed.

**Cell Surface Protein Biotinylation**

Confluent monolayers of Caco-2 and HT-29 control and integrin α5-transfected cells cultured on Transwell polycarbonate membranes were washed twice with ice-cold PBS. 4 °C PBS (pH 8) containing EZ-Link Sulfo-NHS-LC-biotin (Pierce) was added to the apical and basal chambers of the cells and left for 1 h at 4 °C. The cells were washed twice with ice-cold PBS and then once with ice-cold 50 mM Tris-HCl (pH 8) to quench the reaction. The cells were warmed to 37 °C to allow HER-2 endocytosis to resume. At various time points thereafter, the cells were washed and lysed in lysis buffer. The lysates were normalized for total protein concentration and then incubated with 50 μl of a 50% slurry of streptavidin beads in PBS (Pierce) at 4 °C on a rocker overnight. The lysates were aspirated off, and the beads were washed twice with ice-cold PBS and boiled in reducing sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 2% β-mercaptoethanol, 10 μg/ml bromophenol blue) for 3 min. Western blotting for HER-2 were performed as described elsewhere in this section.

**Immunoprecipitation**

Confluent monolayers of cells were lysed in 4 °C lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 100 mM NaF, 10 mM Na2PO4, 1 mM Na3VO4, 10% glycerol, 1% Triton X-100, and 1 μg/ml each of aprotinin, leupeptin, chymostatin, and pepstatin) and clarified at 12,000 rpm at 4 °C for 15 min. The lysates were then normalized for protein concentration to a total volume of 1 ml in lysis buffer. 1–2 μg of primary antibody were added to each tube of lysate, which was then incubated on a rocker at 4 °C overnight. The lysates were washed once with lysis buffer and boiled in reducing sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 2% β-mercaptoethanol, 10 μg/ml bromophenol blue) for 3 min. Western blots of HER-2 were detected with a monoclonal antibody served in cells overexpressing HER-2 (20), the entire HER-2 levels of HER-2 were examined. The integrin α5-transfected Caco-2 and HT-29 cells demonstrated much lower HER-2 protein levels than the control cells (Fig. 1E). Multiple Caco-2 integrin α5 transfectants were isolated and studied to determine the potential effect of clonal selection on HER-2 expression. Three additional stable integrin α5-transfected Caco-2 clones showed HER-2 down-regulation (Fig. 1F, left panel, lanes 4–6), whereas three additional control transfectants failed to show HER-2 down-regulation (Fig. 1F, left panel, lanes 1–3). This showed that the stable expression of integrin α5/β1 consistently resulted in decreased HER-2 expression. Because HER-2 cleavage was observed in cells overexpressing HER-2 (20), the entire HER-2 Western blot, which was performed with a monoclonal antibody that detects the carboxyl-terminal portion of HER-2, is shown (Fig. 1F). No HER-2 cleavage products were detected in the lysates generated from integrin α5-transfected Caco-2 cells.

**Mechanism of Integrin α5/β1-mediated Decrease in HER-2 Expression**—The possibility that integrin α5/β1 caused a decrease in c-erbB2 transcription was examined. Neither HT-29 nor Caco-2 cells that were stably transfected with integrin α5 showed decreased HER-2 mRNA levels compared with the control transfected cells (Fig. 2A). Loading controls of the cell lines showed similar levels of GAPDH mRNA in the samples.

The data in Fig. 2A suggested that integrin α5/β1-induced down-regulation of HER-2 may occur at the post-transcriptional level. To test this hypothesis, Caco-2 cells were transiently transfected with integrin α5. Forty-eight hours after transfection, the cells were lysed and used to generate integrin α5 and HER-2 immunoblots. Down-regulation of HER-2 protein did not occur in Caco-2 cells transiently transfected with integrin α5 (Fig. 2B), but instead it took at least two passages in the presence of the selection agent G418 to see at least a 50% decrease in HER-2 expression compared with the control cells (Fig. 2C). The diminished expression of HER-2 persisted with subsequent passages. The HER-2 protein levels remained constant in the Caco-2 cells stably transfected with vector alone (Fig. 2C). Co-transfection of HER-2 and integrin α5 did not induce apoptosis (data not shown), arguing against a selection mechanism for the emergence of a population of cells expressing integrin α5/β1 and not HER-2.
Integrin α5/β1 Stimulates HER-2 Protein Degradation—One possible explanation for the decreased HER-2 protein levels in the integrin α5/β1-expressing cells was a decrease in the half-life of HER-2 protein. To study this, Caco-2 cells that were transfected with integrin α5 and only passaged once in G418, so they expressed readily detectable levels of both integrin α5 and HER-2 proteins, and control cells were surface biotinylated at 4 °C for 1 h. The cells were then brought to 37 °C to allow receptor endocytosis to resume, and at various time points thereafter, the cells were lysed. The lysates were normalized for total protein concentration and incubated with streptavidin beads. The resulting precipitates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose, and blotted with anti-HER-2 antibodies. The immunoblots show that surface HER-2 protein levels did not change much over the 24-h time course in the control cells, whereas they fell greatly after 12 h in the integrin α5-transfected cells (Fig. 3A). These data showed that the half-life of surface HER-2 receptors was significantly reduced in cells stably expressing integrin α5/β1.

The EGFR is well known to undergo lysosomal targeting and degradation following ligand binding. HER-2 undergoes rapid lysosomal degradation following treatment with HER-2 antibodies but is otherwise endocytosis deficient (21, 22). We hypothesized that the significantly decreased half-life of HER-2 in the integrin α5/β1-expressing cells could be due to increased lysosomal targeting. To test this hypothesis, HT-29 control and integrin α5-transfected cells were treated for various lengths of time with the lysosomal inhibitor chloroquine. Although the control cells showed little change in HER-2 protein levels after 48 h of treatment with chloroquine, the integrin α5/β1-expressing cells demonstrated a large increase in HER-2 protein (Fig. 3B). Thus, the inte-
creased down-regulation of HER-2 in the integrin α5/β1-expressing cells could be reversed by lysosomal inhibition.

As further evidence of increased lysosomal targeting of HER-2 in the integrin α5/β1-expressing Caco-2 cells, a HER-2-eGFP construct was expressed in control transfected and Caco-2-α5 cells using nucleofection. Expression of the HER-2-eGFP protein in the cytoplasm and cell membrane was seen within 1–2 h after nucleofection of the cells (data not shown). Twenty-four hours after nucleofection, HER-2-eGFP was still seen in the cell membranes of Caco-2 control transfected cells (Fig. 3D) but not the Caco-2-α5 cells (Fig. 3G). HER-2-eGFP was more strongly localized to lysosomes in the cytoplasm but absent from the cell membranes of Caco-2-α5 (Fig. 3E and F), whereas Caco-2 control transfected cells showed retention of cell membrane localization as well as co-localization with lysosomes 24 h after nucleofection (Fig. 3H and I).

To determine which portion of integrin α5 was important for the down-regulation of HER-2, truncation mutants of integrin α5 (Fig. 4A) were transiently expressed in HEK 293 cells. The integrin α5 subunit is comprised of an extracellular domain, a single transmembrane domain, and a short cytoplasmic domain. All three mutant constructs were engineered to express a V5 epitope tag at the carboxyl-terminal end of the peptides. The integrin α5 truncation mutant lacking the extracellular domain (ΔECD) and another lacking both the extracellular and cytoplasmic domains but retaining the transmembrane domain were engineered with Myc epitope tags at the amino termini. To successfully express the transmembrane domain and ΔECD constructs in the cell membrane, the integrin α5 signal sequence was inserted 5′ to the Myc epitope tag sequences.

Immunofluorescence detection of the epitope tags was used to determine whether the constructs were expressed at the cell membrane. The mutant constructs were detected with V5 or Myc antibodies (Fig. 4B). The V5 epitope tags were readily detected in the cell membranes for all three truncation products (Fig. 4C). Expression of the transmembrane domain and Δcyto constructs failed to effect HER-2 down-regulation, but expression of the α5-ΔECD construct did (Fig. 4D). These data suggested that the cytoplasmic domain of integrin α5 was sufficient for mediating HER-2 down-regulation.

Integrin α5/β1 and HER-2 Interact—We hypothesized that integrin α5/β1 and HER-2 may be interacting. HEK 293 cells were transiently co-transfected with integrin α5 and HER-2. HER-2 was readily co-immunoprecipitated with integrin α5 (Fig. 5A) and integrin α5 with HER-2 (Fig. 5B). As was ob-
FIG. 3. Integrin α5/β1 increases HER-2 protein degradation. A, Caco-2 cells stably transfected with vector or integrin α5 were surface biotinylated for 1 h at 0 °C. The cells were brought to 37 °C (time 0) and lysed at various time points thereafter. Biotinylated surface proteins were precipitated with streptavidin beads from aliquots of the lysates normalized for total protein concentration. The precipitates were used to generate HER-2 immunoblots. The graph shows the densitometry results of the Western blots. IP, immunoprecipitation; W, Western blot. B, immunoblot of HER-2 generated from lysates of HT-29 cells transfected with vector (α5−) or integrin α5 (α5+) that were incubated in the presence of 4 μg/ml (final concentration) of the lysosomal inhibitor chloroquine for 0–48 h. All of the lanes contain equal total protein concentrations. The graph shows the densitometry results of the Western blot. C–I, fluorescence microscopy images of Caco-2 control transfected (D–F) or Caco-2-α5 cells (G–I) 24 h after nucleofection with a vector containing a HER-2-eGFP (green) construct. The cells were also incubated with Lysotracker dye (red) that localizes to lysosomes. The white arrows show the cell membranes. Representative cells are shown at 100× magnification.
served in Caco-2 and HT-29 cells, stable co-expression of integrin α5/β1 and HER-2 resulted in lower levels of HER-2 than the control transfected cells (Fig. 5B, lower panel). This was despite the fact that HER-2 alone could be highly overexpressed in HEK 293 cells (Fig. 5E). To demonstrate an interaction between HER-2 and integrin α5/β1 in colon cancer cells, HER-2 was co-immunoprecipitated with integrin α5 from lysates of Caco-2 cells stably transfected with integrin α5 (Fig. 5C). These data showed that HER-2 and integrin α5/β1 were interacting in the cells studied, although it is presently unknown whether this interaction is direct or indirect.

Ligand or anti-HER-2 binding to the EGFR and HER-2, respectively, causes activation of the receptors followed by c-Cbl-mediated monoubiquitination of the receptors, which causes lysosomal targeting of the proteins (23, 24). Bands lower in molecular weight were seen on the HER-2 Western blots of HER-2 immunoprecipitates generated from cells singly transfected with HER-2 and to a greater degree from cells co-transfected with integrin α5 and HER-2 (Fig. 5D, open arrows). This suggested the presence of HER-2 degradation products in the cell overexpressing HER-2. Fig. 5E shows the successful overexpression of integrin α5 in the transfected cells.

**DISCUSSION**

During the transformation of normal colonic epithelium to adenocarcinoma, a consistent loss of integrin α5/β1 expression occurs (18). Because HER-2 is commonly overexpressed in colorectal cancers, we examined whether a relationship between integrin α5/β1 and HER-2 expression existed. We showed in the present study that re-expression of integrin α5/β1 in colon...
cancer cells decreased HER-2 protein levels through increased lysosomal degradation.

Zutter et al. (31, 32) previously showed that integrin α2/β1 re-expression in breast cancer cells resulted in decreased HER-2 expression through transcriptional inhibition of c-erbB-2. Another study recently showed that increased signaling through integrin α6/β1 resulted in ectodomain cleavage of c-erbB-2 (20). We did not, however, observe HER-2 cleavage products using a carboxyl-terminal specific HER-2 antibody in colon cancer cells transfected with integrin α5 (Fig. 1F). When HER-2 was overexpressed alone or with integrin α5 in HEK 293 cells, protein bands lower in molecular weight than full-length HER-2 were detected with a carboxyl-terminal and HER-2-specific antibody, suggesting the presence of HER-2 cleavage products (Fig. 5E). It is important to note that colon cancer cells express on the order of tens of thousands of endogenous HER-2 receptors/cell, whereas cells transfected with HER-2 express an order of magnitude or more of HER-2 receptors/cell (9, 33). In the study by Shimizu et al. (20), integrin α5/β1-mediated HER-2 cleavage was observed in cells transfected with HER-2. Thus, HER-2 cleavage has only been observed by others and us in cells that have been transfected with HER-2 or in SKBr-3 cells that express very high levels of HER-2 (20, 34–36). We conclude that integrin α5/β1-mediated cleavage of HER-2 was not an important mechanism in the down-regulation of endogenous levels of HER-2 in colon cancer cells transfected with integrin α5. Alternatively, our results indicate that integrin α5/β1 mediated the down-regulation of HER-2 protein through increased lysosomal targeting of HER-2.

HER-2, HER-3, and HER-4 are all endocytosis-deficient compared with the EGFR (21). HER-2 is unique in that it lacks a ligand but can be stimulated to undergo endocytosis by a variety of antibodies targeting its extracellular domain (24). HER heterodimers are preferentially trafficked to lysosomes whereas heterodimers are recycled back to the cell membrane by the endocytic cellular machinery (37). The crystal structure of HER-2 bound by the Fab portion of mAb 4D5 (trastuzumab) predicted that the complete antibody would cross-link HER-2 molecules and produce homodimers (38). Our finding that integrin α5/β1 was found in complexes with HER-2 suggests that integrin α5/β1 could aggregate HER-2 receptors favoring their endocytosis.

Recent studies have shed light on the regulation of cell membrane integrins. In mammary epithelial cells, β1 integrins were internalized and endocytosed in a calcium-, phosphatidyl-
Inositol 3-kinase-, and dynamin-dependent fashion (39). In fact, integrin β1 co-localized with transferrin receptors within endosomes prior to recycling to the cell membrane. This behavior is very reminiscent of the trafficking pattern of EGFRs following ligand activation. Following activation of the EGFR or HER-2 by ligand or cross-linking antibodies, respectively, the receptors homodimerize, are rapidly autophosphorylated and monoubiquitinated in a C-Cbl-dependent fashion (23, 24). Monoubiquitination of the receptors signals trafficking to multivesicular bodies where they can be recycled to the cell membrane or trafficked to lysosomes. The continued contact of C-Cbl with the monoubiquitinated EGFR is necessary for the final lysosomal targeting step (40).

It is intriguing that β1 integrins activated by stimulatory antibodies traffic to multivesicular bodies in human mammary epithelial cells (39) because it appears to be the decision point for EGFR trafficking to recycling endosomes or to the lysosomes for degradation. Our studies showed that the cytoplasmic domain of integrin α5 was sufficient to cause the down-regulation of HER-2 in colon cancer cells, but little is known about which proteins interact with this domain. A previous study showed that the integrin α5 cytoplasmic domain was important for cytoskeletal organization, a process that is important to receptor trafficking, and other studies have demonstrated the phosphorylation of C-Cbl following integrin ligation (41–44). We do not yet know whether integrin α5/β1 may recruit C-Cbl, or another E3 ligase, and bring it into contact with HER-2 receptors.

Thus, stable integrin α5/β1 expression in colon cancer cells lacking its expression inhibited HER-2 signaling, which could help explain how integrin α5/β1 acts as a tumor suppressor in colon cancer cells. It should be noted that expression of integrin α5/β1 in a breast cancer cell line restored transforming growth factor β1 receptor levels and their susceptibility to transforming growth factor β-mediated inhibition of cell proliferation (46). This mechanism is unlikely to explain the anti-tumorigenic effects of integrin α5/β1 expression in Caco-2 or HT-29 colon cancer cell lines because they both possess inactivating SMAD4 mutations that prevent them from responding to transforming growth factor β receptor signaling (47).

Integrin α5/β1 normally mediates strong survival signaling in response to fibronectin (48). Thus, it seems paradoxical that colon cancer tumorigenesis would favor the loss of integrin α5/β1 expression. The effects induced by the extracellular matrix on normal intestinal epithelial cells also create selective pressures for colon cancer cells, as evidenced by the characteristic ability of the latter to invade through the underlying extracellular matrix. Perhaps the loss of integrin α5/β1 by colon cancer cells is a manifestation of their gain of independ-ence from fibronectin for critical cell functions, such as cell survival, proliferation, and adhesion. Increased growth factor receptor survival signaling may be sufficient to substitute for and thus permit the loss of integrin α5/β1, which normally provides strong survival signaling (48, 49). Indeed, the proliferation and survival of integrin α5-transfected colon cancer cells were much less inhibited by antagonistic EGFR antibodies than the parental cell line (13). Our results suggest too that integrin α5/β1 may function to control HER signaling in normal intestinal epithelial cells.

Future studies should focus on how integrin expression is regulated in cancer cells and to see whether modulation of protooncogene expression caused by alterations in integrin expression is a general feature of cancer cells. Studies of integrin regulation in cancer cells may lend insight into the mechanisms leading to invasion and metastasis.