Apc Deficiency Is Associated with Increased Egfr Activity in the Intestinal Enterocytes and Adenomas of C57BL/6J-Min/+ Mice*

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Overexpression of the epidermal growth factor receptor (EGFR) and its increased tyrosine kinase activity are implicated in colorectal cancer (CRC) development and malignant progression. The C57BL/6-J-Min/+ (Min/+) mouse is a model for CRC and develops numerous intestinal adenomas. We analyzed the normal mucosa of Min/+ and Apc+/+ (WT) littermate mice together with Apc-null adenomas to gain insight into the roles of Egfr in these intestinal tissues. Protein analyses showed that Egfr activity was highest in the tumors, and also upregulated in Min/+ relative to WT enterocytes. Expression of ubiquitylated Egfr (Egfr-Ub) was increased in Min/+ enterocytes and tumors. Tumors exhibited increased association of Egfr with clathrin heavy chain (CHC), Gab1, and p85α, the regulatory subunit of phosphoinositide 3-kinase (PI3K), and tumors also overexpressed c-Src, PDK1, and Akt. Immunohistochemistry for Akt-p-Ser473 revealed a low level of this active kinase in Min/+ and WT enterocytes and its strong presence in tumors. Prostaglandin E2 (PGE2) is a product of cyclooxygenase-2 (Cox-2) activity that is up-regulated in Min/+ tumors and transactivates Egfr. PGE2 expression was significantly higher in untreated Min/+ tumors and reduced by treatment with the Cox-2 inhibitor, celecoxib. Dietary administration of this NSAID also inhibited Egfr activity in tumors. Increased activation of the EGFR-P13K-Akt signaling pathway in tumors relative to Ap+/+ and ApcMin/+ enterocytes provides potential opportunities for therapeutic interventions to differentially suppress tumor formation, promotion, progression, and/or recurrence.

Tumor formation in the lower intestine is a multifactorial process, involving chronic derangement of signal transduction pathways, sequential losses of tumor suppressor genes, and activation of oncogenes (1). The majority of sporadic and familial forms of CRC are associated with initiating mutations in the tumor suppressor gene, adenomatous polyposis coli (APC) (2). Our goal is to understand the earliest events in intestinal tumorigenesis, as it may be possible at this stage to intervene with chemopreventive drugs that suppress tumor formation or progression. As a model for early Apc-associated tumorigenesis, we examined the Min/+ mouse, an animal that develops numerous intestinal adenomas as a result of germline mutation of Apc (3). Adenomas arising in Min/+ mice (ApcMin−/−) uniformly sustain loss of heterozygosity of the remaining Apc+ allele and are consequently devoid of Apc function (4, 5). Previous work demonstrated the inhibition of adenoma formation in these mice by administration of various chemopreventive compounds, including nonsteroidal anti-inflammatory drugs (NSAIDs) (6–8), plant phenolics (9, 10), phytoestrogens, and exogenous estrogens (11).

Ligand-dependent activation of EGFR plays an important role in controlling the proliferation, survival, and migration of epithelial cells. EGFR activity also stimulates the promotion and progression stages of human CRC (12). Several recent studies demonstrated the relevance of EGFR signaling to early Apc-dependent tumorigenesis. For instance, transgenic Min/+ bearing a single impaired allele for the receptor yielded 10-fold fewer tumors than Egfr+/−/− Min−/+ , and a similar result was observed following treatment of Min/+ with a selective EGFR inhibitor, EKI-785 (13). Synergistic inhibition of Min−/+ tumor multiplicity was found in another study that examined the effect of a combination chemoprevention regimen with the NSAID, sulindac, and another selective EGFR inhibitor, EKI-569 (14). NSAIDs likely achieve tumor prevention by inhibition of Cox-2, a well established tumor promoter in mouse models of CRC (15) and in human tumors (16, 17). Cox-2 activity includes the inflammatory prostaglandin, PGE2, and the enhanced efficacy of NSAIDs in combination with receptor-tyrosine kinase (RTK) inhibitors (14) may relate to the finding that PGE2 can transactivate EGFR in intestinal epithelial cells (18, 19).

EGFR regulation is complex. This receptor has multiple ligands and must homodimerize or heterodimerize with other Erb-B family members in the plasma membrane. Growth factor-

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1 The abbreviations used are: APC/Apc, adenomatous polyposis coli; EGFR, epidermal growth factor receptor; RTK, receptor-tyrosine kinase; IB, immunoblot; IP, immunoprecipitation; PNL, post-nuclear lysate; TCL, total cell lysate; PBS, phosphate-buffered saline; CHC, clathrin heavy chain; ALLN, N-acetyl-Leu-Leu-norleucinal; P13K, phosphoinositide 3-kinase; NSAID, nonsteroidal anti-inflammatory drug; Cox-2, cyclooxygenase-2; CRC, colorectal cancer; p-Y, anti-phosphotyrosine antibody; Gab1, Grb2-associated-binding protein 1; Cbl, Casitas B-lineage lymphoma; sPLA2-IIA, secretory type II phospholipase A2; WT, wild type; ELISA, enzyme-linked immunosorbent assay; PGE2, prostaglandin E2.

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Increased EGFR Activity in Min/+ Enteroocytes and Adenomas

EXPERIMENTAL PROCEDURES

Materials—5–6-week-old C57BL/6J Min/+ and WT littermate mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were fed AIN-76A diet with or without cellcoaltar at 1,500 mg/kg (Research Diets, Inc., New Brunswick, NJ). Anti-EGFR (clone 13), e-Chl (clone 17), adapter (clone 8), Gαs (clone 81), and phosphotyrosine (4G10) antibodies and EGF-stimulated A431, NIH3T3, Jurkat, and HeLa-positive control lysates were from BD Biosciences (San Diego, CA). Rabbit anti-phospho-Akt (Ser-473) (9271 and 9277), Akt, EGFR, PKD1, phospho-PDK1 (Ser-414) antibodies were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-β-actin (clone 13) antibody was from BIOSORP International (Charlottesville, CA). Anti-ubiquitin antibody (clone U13) antibody was from BIO-RAD (Hercules, CA). Anti-Rabbit anti-Gab1, Src (GD11), and phosphotyrosine (4G10) antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ubiquitin antibody (clone 17), adaptin (clone 228), and phosphotyrosine (4G10) antibody was from Sigma. Biotinylated molecular size markers were obtained from Jackson Laboratories (Bar Harbor, ME). All animal procedures were performed at 4 °C. For immunohistochemistry (IHC), tissues were washed with Dulbecco’s PBS containing physiological concentrations of MgCl2 and CaCl2. When harvesting Min/+ tissue, intestinal segments were opened longitudinally, and adenomas were quickly counted, excised, and pooled. Tumors were then set on ice for 1 h. Tissue pellets were frozen and stored at −80 °C. In parallel, enterocytes from the WT and Min/+ small intestine were harvested using the edge of a glass slide, a microscope slide, and a 10 mm P100 filter. Tissue pellets were frozen and stored as above. Pervanadate (10 mM) was freshly prepared as detailed (42) and added to warm Dulbecco’s modified Eagle’s medium. Opened, washed segments of small bowel were immersed in this medium with and without the treatment agent, and placed in an humidified 5% CO2 incubator at 37 °C for 15 min, prior to enterocyte collection as above. Treatments with PGE2 (200 mM) and AG1478 (70 μM) were also performed in this manner.

Increased EGFR Activity in Min/+ and WT mouse intestines—opened, washed segments of small bowel were homogenized with 30 strokes. Cell lysates were clarified by centrifugation at 14,000 rpm for 10 min. Aliquots were removed for protein determinations. For immunoprecipitations, lysates were preincubated with 2 ml of buffer for 3 h with agarose G-beads. These beads were removed, and immunoprecipitations were performed. Consequently, small intestine was first homogenized with 30 strokes. Cell lysates were spun at 1,700 rpm for 5 min in the cold. Washed enterocytes were either used immediately to prepare protein lysates or cell pellets were frozen and stored as above. Permeate was then set on ice for 1 h. G-beads were again added, and samples were rocked overnight. After G-bead removal, stringent washing and eluting was performed as detailed by the vendor. To separate proteins into membrane (P100) and cytosolic (S100) fractions, post-nuclear lysates were prepared (41). Briefly, enterocytes were placed in Dounces containing 4 ml of a detergent-free lysis buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM Mg(Ac)2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM ALLN, 3 mM H2O2, and 1% Triton X-100). Pooled tumors (~30–50 tumors per sample) were placed in 0.5 ml of this buffer. WT and Min/+ samples were homogenized for 10 tumors and for 30 strokes. Lysates were clarified by centrifugation at 14,000 rpm for 10 min. Aliquots were removed for protein determinations. For immunoprecipitations, lysates were preincubated with 2 ml of buffer for 3 h with agarose G-beads. These beads were removed, and immunoprecipitations were performed. Consequently, small intestine was first homogenized with 30 strokes. Cell lysates were spun at 1,700 rpm for 5 min in the cold. The supernatant were then centrifuged in a T70.1 rotor at 100,000 × g for 1 h. The S100 supernatants containing cytosolic proteins were removed to separate tubes and mixed with Laemmli sample buffer. The P100 pellets containing intact and associated membrane proteins were also suspended in this buffer. Aliquots of both fractions were collected for protein determinations, and the samples were stored at −80 °C. Immunoblot analyses were performed as previously detailed (40). In every experiment shown, the input TCLs from the same preincubated samples used for immunoprecipitations were analyzed in parallel. Previously filtered membranes were placed in stripping buffer (88 mM Tris·HCl, pH 6.8, 10% SDS, 0.1% β-mercaptoethanol) at 65 °C for 1 h. The membranes were then set on ice for 10 min, then homogenizing with 30 strokes. Cell lysates were spun at 1,700 rpm for 5 min in the cold. The supernatant were then centrifuged in a T70.1 rotor at 100,000 × g for 1 h. The S100 supernatants containing cytosolic proteins were removed to separate tubes and mixed with Laemmli sample buffer. The P100 pellets containing intact and associated membrane proteins were also suspended in this buffer. Aliquots of both fractions were collected for protein determinations, and the samples were stored at −80 °C. Immunoblot analyses were performed as previously detailed (40). In every experiment shown, the input TCLs from the same preincubated samples used for immunoprecipitations were analyzed in parallel. Previously filtered membranes were placed in stripping buffer (88 mM Tris·HCl, pH 6.8, 10% SDS, 0.1% β-mercaptoethanol) at 65 °C for 20 min before re-probing. All experiments were repeated using independently prepared lysates from separate animals at least three times. PGE2, ELISA—Washed enterocytes from one-half of the small intestines of WT and Min/+ mice and ~40 pooled Min/+ adenomas were separately lysed in 1 ml of radioimmune precipitation assay buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, and 0.5% NP-40). The lysates were then separated into membrane (P100) and cytosolic (S100) fractions by homogenization and clarified, as above. PGE2 measurements were performed using the vendor’s protocol. Briefly, aliquots (3 mg) of each lysate were subjected to PGE2, affinity chromatography for 1 h at 4 °C. Bound proteins were eluted with 95% ethanol that subsequently was evaporated by vacuum centrifugation. The resulting eluates were each subjected to 90% ethanol precipitation reactions of samples, diluted 1:20 and 1:50 in EIA buffer, were run in parallel with a standard curve. Absorbance at 405 nm was measured in a Molecular Devices microplate reader (Sunnyvale, CA).

Immunohistochemistry—For IHC, serial 4 μm sections of tumor-
bearing intestinal mucosa from Min/+ mice were deparaffinized and rehydrated. Antigen retrieval was performed with 10 mM citrate buffer pH 6.0 in a pressure cooker for 10 min. Endogenous peroxidases were quenched with 3% H2O2 in methanol, and slides were then washed in PBS. The sections were incubated with rabbit anti-EGFR (1:50) or anti-Akt (1:200) antibody for 1 hour at room temperature. Slides were then washed in PBS, and sections were incubated in EnVision 228 System anti-rabbit labeled polymer for 30 min at room temperature. Enzyme detection was performed with DAB chromogen. Sections were counterstained with hematoxylin, dehydrated, and coverslipped. Images were obtained using an Olympus BX40 microscope at 60× power.

RESULTS

Egfr Activation Is Increased in ApcMin/+ Enterocytes and ApcMin/+ Intestinal Adenomas—Because the Apc allele perturbs enterocyte adhesion and migration in the intestine (41, 43), we hypothesized that Egfr activity is increased in both the normal mucosa and adenomas of Min/+ mice relative to the WT littermate. Therefore, we assessed the overall level of Egfr expression in TCLs of WT enterocytes and tumors obtained from adult Min/+ mice. Immunoblot analysis (Fig. 1A) using EGFR antibody (clone 13) showed that overall Egfr expression was increased in tumors relative to WT enterocytes. By our lysis and electrophoresis conditions, Egfr protein in the mouse samples yielded several bands in a size range of 170–200 kDa. The 200-kDa band was especially prominent in tumors when compared with untreated WT. A similar size range was evident in the commercially obtained, positive control lysate (EGF-stimulated A431). In this and subsequent figures, immunoblots of the TCLs used for each of the immunoprecipitations were included, and immunoprecipitation and sample loading controls were detailed in the legends.

The amount of tyrosine-phosphorylated EGFR (EGFR-p-Y) expressed in cells is an indicator of its activation level. To compare the relative expression of Egfr-p-Y in WT tissue to that present in tumors, we performed reciprocal immunoprecipitations followed by immunoblots using 4G10 anti-phosphotyrosine (p-Y) and EGFR antibodies (Fig. 1B). To further assess
the differential expression of Egfr in our specimens, we performed ex vivo treatment of WT intestinal tissue with pervanadate, a cell permeable tyrosine phosphatase inhibitor that stimulates overall protein tyrosine phosphorylation (44). When TCLs of WT tissue treated with pervanadate were used for immunoblots, Egfr bands of ~170 and ~200 kDa were intensified and resembled those produced from the adenoma lysates (Fig 1A, lane 3). As anticipated, pervanadate treatment of WT tissue increased Egfr expression. In immunoprecipitation experiments to compare the relative amounts of Egfr tyrosine phosphorylation, a single band of ~170 kDa was detected in the untreated WT enterocytes, however, ~200- and ~170-kDa bands appeared in the pervanadate-treated WT and tumor samples. As expected, active Egfr was present in the normal WT mucosa (Fig. 1B). Because the ~200-kDa band occurred in both the Egfr and p-Y immunoprecipitations of pervanadate-treated WT and tumor samples, this band may account for the overall increase of Egfr in tumors relative to untreated WT. This band also represents a tyrosine-phosphorylated form of Egfr.

Egfr activity was attributed to stimulate the growth and/or progression of intestinal adenomas at the very earliest stages (13). Since all adenomas in the Min/+ mouse are Apc-null due to loss of heterozygosity (4), this idea implied that the complete loss of Apc function in polyps precedes Egfr dysregulation. To examine this, we compared the expression and activity of Egfr in lysates of Min/+ enterocytes to those of WT littermates. Immunoblot analysis of normalized amounts of protein presented in Fig. 1C (top) revealed higher Egfr expression in Min/+ relative to WT enterocytes. Reciprocal immunoprecipitations and immunoblots of these samples using the EGFR and 4G10 antibodies were performed to assess the relative activity of Egfr. Greater amounts of Egfr-p-Y were present in Min/+ than in WT enterocytes (Fig. 1D, middle). To confirm and extend these data, we performed immunoblot analyses using WT, Min/+ , and tumor-fractionated cell lysates. The P100 fraction contained membrane-integral and -associated proteins, while the S100 fraction contained cytosolic proteins. Immunoblots were probed with two different phospho-specific antibodies directed against active, human EGFR (Fig. 1D). Each of these antibodies recognizes the mouse homolog of this receptor. Significant amounts of membrane-localized, active Egfr were present only in tumors. Unlike the WT sample, however, an appreciable amount of active Egfr was present in the soluble fractions of both normal Min/+ enterocytes and adenomas. This result suggests that in Apc+/− cells, Egfr protein is efficiently down-regulated, but that negative regulation of this receptor kinase is deficient in Min/+ enterocytes and tumors.

Binding of the growth factor, EGF, to EGFR occurs in the basolateral membranes of intestinal enterocytes (45), however 80–85% of total immunoreactive EGFR is associated with intracellular organelles (46). We performed IHC to examine the expression and localization of Egfr within the crypt-villus axis of WT proximal ileum treated with and without pervanadate. Using a rabbit anti-EGFR antibody, positive staining was evident in the WT small intestine, especially in mid- to upper-villus enterocytes (Fig. 2A, left). As expected, Egfr was diffusely present in the cytoplasm in the untreated, normal enterocytes. This result suggested that active Egfr is internalized in WT cells either for recycling to the plasma membrane or turnover. Egfr IHC of the WT tissue treated with pervanadate that was performed in parallel showed strongly enhanced immunostaining, especially at apical and basal membranes (Fig. 2A, right). These results indicated that our conditions for ex vivo pervanadate treatment increased Egfr expression in WT enterocytes, yet our treatment conditions maintained normal tissue integrity. We then performed Egfr IHC using sections of Min/+ and WT ileum in parallel. Fig. 2B showed that overall expression of Egfr was increased in Min/+ (right) relative to WT (left) enterocytes and confirmed the results of Fig. 1, C and D. Positive staining for Egfr in the Min/+ tissue was also cytoplasmic. However, levels of basal and apical staining were increased in Min/+ relative to WT. This result suggested that ApcMin/+ enterocytes also internalize ligand-activated Egfr. Images of all of these small intestine specimens were cut perpendicular to the crypt-villus axis and showed that Egfr expression was greater in cells of the upper villi relative to the crypts.

Evidence for Increased Internalization of Egfr Confirms That Its Activity Is Increased in ApcMin/+ Enterocytes and ApcMin/+--Intestinal Adenomas—Internalization of ligand-bound EGFR via endocytosis initiates its down-regulation. Active EGFR is taken into clathrin-coated pits from the plasma membrane, and may remain associated with its ligand until its degradation in lysosomes (47–49). The association of EGFR with CHC is ligand-dependent, and therefore is another assay of this receptor’s activity (50). We performed immunoblot analysis to assess the relative expression of CHC in our TCLs from WT and Min/+ enterocytes and tumors. We found increased CHC expression in the tumors relative to the two mucosal samples (Fig. 3A, top). Immunoblots of fractionated proteins from WT, Min/+ , and tumor lysates showed increased association of CHC with membranes in the tumors but not in either Min/+ or WT mucosa (Fig. 3A, bottom). This result was consistent with the overexpression of CHC observed in the tumor TCL. Consistent with changes in the amount of CHC in the P100, α-adaptn, the AP-2 adaptor subunit required for clathrin to associate with membranes (51, 52), also was increased in the P100 fraction of the tumor relative to that of WT and Min/+ .

We next examined the association of CHC with Egfr and the relative tyrosine phosphorylation of CHC in WT enterocytes and tumors. Because the expression of CHC was similar in WT and Min/+ enterocytes, the latter was excluded from these experiments. Fig. 3B shows the immunoblots of reciprocal CHC (top) and Egfr (bottom) immunoprecipitations. In both experi-
ments, a greater association of Egfr with CHC was observed in adenomas when compared with the WT sample. The tyrosine kinase, c-Src, phosphorylates CHC (50), and its overexpression accelerates clathrin-mediated internalization of active EGFR (53). A separate immunoblot of proteins precipitated with anti-CHC antibody was probed with anti-p-Y antibody (Fig. 3B, middle) and showed that levels of CHC-p-Y were increased in adenomas relative to WT. Further immunoblot analysis revealed increased expression of c-Src in adenomas relative to WT enterocytes (Fig. 3C). These data correlate increased Egfr activity and membrane-localized CHC in tumors with up-regulated c-Src expression.

Monoubiquitylation of EGFR induced by c-Cbl is sufficient for its internalization from the plasma membrane and transit to lysosomes for degradation (23, 54). The addition of at least four ubiquitin chains (polyubiquitylation) is required to target substrates including EGFR to the 26 S proteasome for degradation (55). To further assess the relative activity of Egfr in Min/+ and tumors and to learn more about the in vivo regulation of this RTK in intestinal tissue, immunoblot analysis for c-Cbl was performed (Fig. 4A). In TCLs of Min/+ enterocytes (right), but not those of tumors (left), c-Cbl was overexpressed relative to WT. Immunoblot analysis also was performed using fractionated cell lysates (Fig. 4B). In agreement with the elevated level of c-Cbl in the Min/+ TCL, we observed an increased amount of this protein in the cytosolic fraction of Min/+ enterocytes. We identified increased association of these proteins in Min/+ relative to WT (Fig. 4C). As a final test to confirm that Egfr activity was up-regulated in both Min/+ enterocytes and tumors, we compared the relative amounts of ubiquitylated Egfr (Egfr-Ub) in these samples with that of WT. As shown in Fig. 4D, greater amounts of Egfr-Ub were observed in tumors (top) and in Min/+ (bottom) when samples were immunoprecipitated with EGFR antibody and the immunoblots were reacted with anti-ubiquitin antibody. Since only the active receptor is ubiquitylated, this result is consistent with the data of Figs. 1 and 4. The size of the predominant Egfr-Ub band was ~170 kDa in all of the samples, suggesting that it contained Egfr that was monoubiquitinated. The

Fig. 3. Relative CHC associations with EGFR and c-Src expression in WT small bowel and Min/+ adenomas. A, immunoblots (IB) of TCLs (top) and fractionated cell lysates (bottom) prepared from WT enterocytes and Min/+ tumors used the anti-CHC antibody, clone 23 at 1:2,000 dilution. For blots of TCLs, 50 μg protein per lane was used and for those of fractionated samples, 20 μg. The immunoblot shown below of fractionated samples used the anti-α-adaptin antibody, clone 8, at 1:1,000 dilution. HeLa cell lysates were included as a positive control for CHC and α-adaptin proteins. Loading controls were performed as described in the legend to Fig. 1, above. B, immunoprecipitations (IP) and immunoblots assessed the relative association of CHC with Egfr and the expression of CHC-p-Y in TCLs of WT enterocytes and Min/+ tumors. Immunoprecipitations using anti-CHC antibody were performed with 500 μg of protein (B, middle) and those using anti-EGFR antibody (B, top) were performed as detailed in the legend to Fig. 1. The entire amount of each sample was used for immunoblot analyses with anti-CHC and EGFR antibodies, respectively. As controls for the EGFR and CHC immunoprecipitations, the EGFR immunoblot was re-probed for CHC and α-adaptin (B, middle and bottom). C, immunoblot analysis for c-Src of TCLs from WT enterocytes and tumors. Immunoblots were of 50 μg of protein per lane, and the anti-c-Src antibody used was clone GD11 at 1:2,000. NIH3T3 lysate was included as a positive control for c-Src protein. The membrane was cut horizontally and the bottom portion was probed for β-actin to control for sample loading.
200-kDa band, detected in the Egfr immunoblots of Fig. 1B, may be a polyubiquitylated species or one variously modified in other ways to account for the increased size.

Increased Activation of Phosphoinositide 3-Kinase (PI3K)-Akt Signaling Pathway in Apc\(^{Min/+}\) Adenomas—We anticipated that signaling downstream of Egfr would differ in adenomas from that of WT and Min/+ tissues. Because the PI3K-Akt signaling pathway is active in colon carcinogenesis (57, 58), we assessed the relative expression, activity, and localization of Akt in WT tissue and tumors. Initial experiments established that there was little or no difference in the expression of Akt in WT and Min/+ (data not shown), therefore subsequent work on this pathway excluded Min/+ (top) and WT and tumor (bottom) lysates prepared in parallel that were immunoprecipitated with anti-Egfr antibody as described above and immunoblotted using the anti-ubiquitin antibody, P4D1, at 1:1000 dilution (D).

Fraction of tumors, whereas in WT enterocytes it was located mainly in the S100 fraction (Fig. 5B). IHC of adenoma-containing Min/+ tissue using a different Akt-p-Ser473 antibody, showed the uniform presence of active Akt throughout this lesion (Fig. 5C). Akt regulates transcription (60), and prominent nuclear staining for this kinase is evident in Fig. 5C. Stromal cells contained active Akt, but minimal positive staining was detected in histologically normal Min/+ absorptive enterocytes and goblet cells of a villus adjacent to the adenoma or in those cells surrounding the lesion.

We next sought to link Akt activation in tumors with upstream active Egfr. PI3K phosphorylates substrates at the 3-position of the phosphoinositol ring to generate PI 3,4-P\(_2\) and PI 3,4,5-P\(_3\) phospholipids at the cell membrane (61). The latter product is necessary to activate both the kinase activator of Akt, PDK1, as well as Akt (59). Immunoblot analysis of TCLs revealed the relative expression of total PDK1 and its active form, PDK1-p-Ser473 in tumors and WT enterocytes (Fig. 5D). Again, both the steady-state expression of PDK1 and levels of the active kinase were increased in tumors relative to WT enterocytes.
Evidence for Sustained Activation of the PI3K-Akt Pathway in Apc\(^{-}\)/+ Adenomas via Up-regulated Gab1 Expression and Increased Gab1-PI3K Association—Upon growth factor-dependent activation of RTKs, Grb2 binds to EGFR followed by the binding of Grb2-associated binder protein 1 (Gab1) to Grb2, and the recruitment of PI3K (62). Activated PI3K produces PI3,4,5-P\(_3\) in the membrane vicinity of EGFR (63). Schlessinger and co-workers (64) described a mechanism for sustained Akt activation that is initiated by EGFR and Grb2. EGFR-evoked PI3K signaling produces PI3,4,5-P\(_3\) in the plasma membrane that consequently allows Gab1 binding via its N-terminal pleckstrin-homology (PH) domain. When Gab1 is anchored in the membrane, it continues to recruit p85\(^\text{\textsubscript{PI3K regulatory subunit}}\) and to prolong or amplify PI3K activity. Given the results of Fig. 5, we predicted that adenoma formation would be associated with sustained rather than transient PI3K activation via Egfr, and therefore examined the relationships between Egfr, Gab1, Grb2, and the PI3K regulatory subunit, p85\(_\text{\textsubscript{PI3K}}\). EGFR phosphorylates Gab1 on multiple tyrosines, and there are three sites on Gab1 that can bind to the SH2 domain of p85\(_\text{\textsubscript{PI3K}}\) (65). To determine whether Gab1 was involved in Akt activation downstream of PI3K activation, we analyzed the expression of Gab1 and its associations with p85\(_\text{\textsubscript{PI3K}}\) and Egfr. Immunoblot analysis for Gab1 (Fig. 6A, top) showed that both expression and membrane association of this adaptor protein were up-regulated in tumors. Additional immunoblots showed that Gab1 expression in Min/\(+\) was low and similar to WT. (data not shown). The increased membrane localization of Gab1 in fractionated lysates of tumors (Fig. 6A, bottom) was consistent with Gab1 binding to PI3,4,5-P\(_3\) in membranes (64). We evaluated the relative expression of the PI3K regulatory subunit, p85\(_\text{\textsubscript{PI3K}}\), in our samples and found similar levels in both adenomas and WT enterocytes (Fig. 6B). Growth factor-dependent EGFR activation induces PI3K activity (66). Precipitating Egfr in the samples and immunoblotting for p85\(_\text{\textsubscript{PI3K}}\) (Fig. 6C, top) showed that the association of these proteins was greater in tumors than in WT enterocytes. Since the activation of PI3K by EGFR is weak compared with activation induced by other RTKs (67), and the Egfr immunoprecipitation showed a substantial association with p85\(_\text{\textsubscript{PI3K}}\) (Fig. 6C), we further examined characteristics of Gab1 in our specimens. An immunoprecipitate of p85\(_\text{\textsubscript{PI3K}}\) from TCLs using U13 antibody and immunoblot analysis for Gab1 showed an increased association of these proteins in adenomas (Fig. 6D, top). Gab1 is tyrosine-phosphorylated by activated RTKs (63, 64, 66), as well as c-Src (65). Precipitation of Gab1 and immunoblot analysis using anti-p-Y antibody showed that expression of tyrosine-phosphorylated Gab1 was greater in adenomas (Fig. 6E, top). Precipitation of Egfr and immunoblot analysis using Gab1 antibody also showed that the association of these proteins was higher in the adenomas (Fig. 6E, middle) consistent with the increased activity of Egfr and increased expression of c-Src in these lesions.

If sustained PI3K-Akt activation does not require EGFR and Grb2 as suggested (64), we predicted that, unlike the associations of Gab1 with p85\(_\text{\textsubscript{PI3K}}\) and Egfr, interactions between Grb2 and Egfr would be equivalent in tumors and WT cells. We performed immunoblotting using fractionated proteins and TCLs (Fig. 7A), and, as expected, found that expression of Grb2 was invariant in these samples. Reciprocal immunoprecipitations and immunoblots to test the associations of Egfr and Grb2 in adenomas and WT enterocytes yielded bands of similar intensities (Fig. 7B). This result is consistent with the view that the EGFR and Grb2 interaction is transient and that the amplification of the PI3K-Akt pathway depends on the membrane docking of Gab1.

NSAID Treatment Induced Down-regulation of Egfr Expression and Inhibition of PGE\(_2\) in Apc\(^{-}\)/- Tumors—Membrane arachidonic acid (AA) is converted by cyclooxygenases to PGG\(_2\) and then to PGE\(_2\) that is subsequently metabolized to PGE\(_2\) by PGE synthase. Basal levels of PGE\(_2\) are normally maintained in the gut by Cox-1 expression, whereas increased levels of this product in neoplastic tissues, as well as adenomas are generated by Cox-2 (68). Because PGE\(_2\) can activate EGFR in vitro (18, 19), we performed an ELISA to quantitate the amount of PGE\(_2\) expression in WT and Min/\(+\) tissue and Apc\(^{-}\)/- adenomas in vivo. For this experiment, we included tumor specimens from –4-month-old Min/\(+\) mice that were fed AIN-76A diet containing 1,500 mg/kg celecoxib, a dose equivalent to the 400 mg b.i.d. regimen currently in use in humans with familial adenomatous polyposis col. Assaying Min/\(+\) enterocytes showed a higher level of PGE\(_2\) expression when compared with these WT cells of littermates (p = 0.003). Significantly elevated...
levels (<15-fold, p = 0.001) of PGE2 were detected in adenomas compared with WT tissue. Tumor PGE2 levels were reduced by ~50% (p = 0.001) following short-term (3 day) celecoxib treatment (Fig. 8A). TCLs were prepared in parallel of the treated and untreated tumors and WT enterocytes and used for immunoblot analyses of Egfr and Gab1 (Fig. 8B). This experiment again showed that expression of both these proteins was constitutively elevated in tumors relative to WT. The Egfr blot was subsequently re-probed with anti-p-Y antibody. Interestingly, Egfr expression was markedly reduced in celecoxib-treated tumors relative to untreated tumors, and tyrosine phosphorylation of proteins between 170–200 kDa was nearly ablated. This result suggests that celecoxib suppressed Egfr activity, and potentially the activities of other RTKs. In addition, Gab1 expression in the drug-treated tumors was substantially inhibited relative to the untreated tumors. These data suggest that Gab1 expression in enterocytes may depend on Egfr activity.

Finally, to assess the relevance of PGE2 to Egfr activity in the mouse intestine, we exposed the small bowel lumen of WT mice ex vivo for 15 min to either Dulbecco’s modified Eagle’s medium alone, or this medium containing PGE2 (200 nM) or AG1478 (70 nM). The latter compound is a highly selective inhibitor of EGFR kinase activity. An immunoprecipitation using anti-p-Y antibody and immunoblot for Egfr showed that exposure to exogenous PGE2 increased the expression of Egfr-p-Y in the normal mucosa and that treatment with AG1478 had the opposite effect (Fig. 8C, top). Additional immunoblot analyses of TCLs showed that PGE2 treatment increased Egfr and Gab1 expression in WT enterocytes. AG1478 treatment strongly suppressed Egfr expression in these cells, but reduced Gab1 to its basal level (Fig. 8C, bottom). These data support the view that PGE2 stimulates Egfr activity in the intestine in vivo.

DISCUSSION

Apc mutation is among the earliest alterations leading to intestinal tumor formation. In adenomas from Min+ mice, Apc
function is lost due to acquisition of a "second hit" mutation in the remaining Apc allele (4, 5). As a result, β-catenin accumulates within the cell, leading to increased transcription of tumor-promoting genes under the control of the β-catenin-Lef-1/Tcf-4 transcription factor (1, 2). This work extends our understanding of Apc-associated tumor formation by showing that Egfr activity is up-regulated in Min/+ enterocytes and tumors when compared with the WT mucosa. Our observations are consistent with the genetic study of Roberts et al. (13) who showed that functional deficiency of Egfr reduced adenoma formation in a strain of Min/+ mice. Fig. 9 summarizes relevant signal transduction pathways examined in this work. We showed that adenomas contained increased levels of Egfr-p-Y, PGE2, and Egfr-Ub (Figs. 1, 8A, and 5C, respectively), as well as increased association of Egfr with CHC (Fig. 3B). The relative levels of Egfr-Ub and Egfr interacting with CHC constitute independent activity assays because only the kinase active receptor is ligated to ubiquitin via interaction with c-Cbl and internalized from the plasma membrane into clathrin-coated pits.

Moreover, we found that the Egfr-PI3K-Akt pathway is constitutively active in tumors, whereas this mode of signaling is minimal and/or transient in histologically normal Min/+ and WT enterocytes (Fig. 5). As indicated by asterisks in Fig. 9, we also showed that several Egfr-associated effectors were overexpressed in tumors relative to Min/+ and WT enterocytes, including c-Src (Fig. 3C), Gab1 (Fig. 6A), PDK1, and Akt (Fig. 5, A and D). In Min/+ enterocytes, we detected a small increase in PGE2 expression relative to WT (Fig. 8A), as well as overexpression of c-Cbl (Fig. 4, A and B).

Fig. 9 also depicts that Egfr activity can be potentiated by H2O2 (69, 70) and c-Src (71, 72), both of which inhibit the function of c-Cbl. Overexpression of c-Src induces c-Cbl turnover resulting in increased Egfr activity (71, 72). A role for H2O2-mediated oxidative stress in our model is supported by extensive evidence that antioxidants prevent intestinal tumorigenesis in Min/+ (9–11). The expression of both c-Src and EGFR are increased in the large majority of CRCs (12, 73), a result we also found in Min/+ adenomas (Fig. 3C). Thus, in combination or separately, c-Src up-regulation and/or the oxidative stress of enterocytes in vivo may constitute mechanisms by which these tyrosine kinases act synergistically to promote CRC (74). Finally, our data support a role for Gab1 in amplifying PI3K signaling in Min/+ adenomas, consistent with the model of Rodrigues et al. (64).

Our observation of elevated PGE2 expression in Min/+ adenomas (Fig. 8A) strongly supports a role for cyclooxygenase activity in adenoma formation. It is also consistent with previous work that showed the elevation of this eicosanoid in Min/+ tissue and its modulation by NSAID treatment (75). Intestinal adenomas in mouse CRC models grow in a microenvironment that contains high Cox-2 activity, likely produced by stromal fibroblasts and endothelial cells (76). Targeted deletion of the murine Cox-2 gene (Figs. 2–4) virtually eliminated Apc-dependent tumor formation (15) and suppression of Cox-2 activity by NSAIDs prevented tumor growth and/or induced their regression in mice and humans (8, 16). Moreover, the important role of PGE2 in promoting adenoma formation in Min/+ mice was emphasized when an E prostaglandin receptor agonist attenuated the suppressive effects of NSAIDs on tumor growth (77). Possible mechanisms for the oncogenic consequences of elevated Cox-2 activity include the stimulation of angiogenesis and the inhibition of apoptosis to support tumor cell growth and survival (78, 79). By various means, the PI3K-Akt pathway also suppresses programmed cell death (60). Celecoxib treatment previously was shown to block Akt activation and to stimulate the apoptosis of certain tumor cells (80, 81). Here, we found that short duration PGE2 inhibition rapidly decreased tumor Egfr expression and activation in vivo (Fig. 8B). Also, exogenous PGE2 treatment of WT tissue enhanced Egfr-p-Y expression (Fig. 8C).

Thus, our data is consistent with a Cox-2-mediated inhibition of apoptosis, via PGE2 activation of the Egfr-PI3K-Akt cell survival pathway in vivo.

PGE2 expression was elevated in the normal mucosa of Min/+ mice relative to the WT tissue (Fig. 8A) and may contribute to the increased activity of Egfr (Fig. 1, C and D). However, increased Egfr activity and PGE2 expression in the normal small intestine of Min/+ may be attributable to various factors. For instance, Min/+ and WT enterocytes may differ in levels of phospholipase A2 (PLA2) activity, leading to differences in liberated AA, the substrate for conversion to PGE2 by cyclooxygenases. Oxidative stress in Min/+ intestinal tissue may induce cPLA2 activation via a mitogen-activated kinase-dependent mechanism, stimulating Egfr and Cox-2 activities and PGE2 production (82–85). Other factors may enhance Egfr activity in the Min/+ small intestine independent of PGE2. Endocytic trafficking may be compromised by deficient Apc function affecting the stability of actin filaments or microtubules at the cell periphery (86) since these cytoskeletal ele-
ments provide the conduits for vesicular transport. The Apc\textsuperscript{Min} allele synergizes with the complete loss of secretory type II phospholipase A\textsubscript{2}, (sPLA\textsubscript{2}-IIA) activity in C57BL/6J mice to accelerate intestinal tumorigenesis (87, 88). Both sPLA\textsubscript{2}-IIA and EGFR accumulate in caveolae and lipid rafts (89, 90). These cholesterol- and sphingolipid-rich membrane microdomains are internalized and transported via their specific trafficking pathways, and sPLA\textsubscript{2}-IIA may be involved in their signaling and sorting fates. Interestingly, sPLA\textsubscript{2} activity inhibited growth factor-induced EGFR activation in A431 cells (91). PLA\textsubscript{2} activity apparently plays no role in the internalization of marker proteins from the plasma membrane, however pharmacological inhibition of PLA\textsubscript{2} completely blocked cytosolic vesicle fusion (92). A defect in vesicle fusions inside enterocytes may slow turnover of active Egfr and account for the presence of membrane-integral and -associated proteins that we detected at increased levels in the S100 of Min/+ fractionated cell lysates (Figs. 1D and 4B, and Ref. 41). It is conceivable that

![Diagram](image-url)
internalized vesicles that are unable to fuse and be sorted eventually break down, releasing their cargo into the cytoplasm. Another possible cause of the increased Egfr activity in Min/+ enterocytes may be transactivation by G protein-coupled receptor ligands other than PGE₂ such as lysophosphatidic acid and neuroendocrine factors (93). Moreover, activation of Egfr may occur upstream of PLA₂ activity and PGE₂ production (94, 95). Finally, we cannot completely rule out the possibility that our enterocyte samples from Min/+ mice may have been contaminated at some low level with microadenomas that contain elevated PGE₂, although the increased expression of Egfr shown in Fig. 2B argues against this possibility.

Because it is a regulator of both proliferation and migration, Apc is likely to have distinct functions in enterocytes depending on their stage of differentiation and position within the crypt-villus unit. For example, Apc permits Wnt signaling in proliferating cells that are located near crypts (96), and this activity is absent in cells located near villus tips. Wnt expression forms an ascending gradient in the crypt-villus axis of the mouse small intestine and is highest at the tips of villi (97). Interestingly, the pattern of Egfr expression in WT proximal ileum (Fig. 2) resembles the distribution of Apc staining. Near villus tips, Apc protein is virtually absent from the cytoplasm and accumulates at the lateral plasma membranes of enterocytes (97). In contrast, we observed Egfr in the cytoplasm and excluded from junctional sites of lateral membranes of cells located at the villus tips (Fig. 2). APC competes with E-cadherin for binding to β-catenin (98), as does Egfr (34). Association of β-catenin with APC targets β-catenin for degradation via the proteasome, thereby preventing β-catenin-mediated Wnt signaling. Conversely, tyrosine phosphorylation of β-catenin by EGFR simultaneously dissociates β-catenin from E-cadherin and promotes the stabilization of β-catenin (36). Hence, Egfr activity expands the pool of free β-catenin and activates β-catenin-Lef/µTcf-dependent gene expression in the absence of cell proliferation (99). The lateral membrane location of Apc in normal mid- to upper-villus enterocytes may exclude Egfr from cell-junctional zones and allow accumulation of this RTK at basal membranes (Fig. 2). The compartmentalization of Egfr would be expected to strengthen E-cadherin-dependent cell-cell junctions. Indeed, E-cadherin-mediated intercellular adhesion negatively regulates EGFR activity in keratinocytes (100).

Our laboratory is interested in applications of small molecule drugs for the prevention of APC-dependent colon polyp formation and recurrence. Drug-induced reduction in the expression and/or activity of some of the EGFR effectors noted in this study can be expected to correlate with these therapeutic endpoints. Certain plant phenolic agents are known to nonselectively inhibit EGFR autophosphorylation (101), and this action may partially account for their tumor prevention efficacy (9). Our data (Fig. 5) suggest that the drug-induced inhibition of signaling upstream of Akt (102) also may be effective in intestinal tumor prevention. Because chemoprevention therapies must be minimally toxic, more work must be done to understand the effects of EGFR pathway inhibitors on the normal intestinal mucosa. For example, the maintenance of proper barrier function in the intestine has been attributed to EGFR activity (103). During the long duration treatment that is typically envisioned for tumor prevention, loss of EGFR activity may have deleterious consequences because its functions are physiologically necessary in the normal gut. Although some studies showed that selective EGFR inhibitors reduced tumor incidence in Min/+ (13, 14), the use of a different compound (PD179851) in this model failed to inhibit tumor number and induced duodenal injury (104). Expression and activity of Egfr is present in WT ent-

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