Raf-1 activation suppresses neuroendocrine marker and hormone levels in human gastrointestinal carcinoid cells

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Submitted 26 September 2002; accepted in final form 3 April 2003

Sippel, Rebecca S., Jennifer E. Carpenter, Muthusamy Kunnimalaiyaan, Sara Lagerholm, and Herbert Chen. Raf-1 activation suppresses neuroendocrine marker and hormone levels in human gastrointestinal carcinoid cells. Am J Physiol Gastrointest Liver Physiol 285: G245–G254, 2003; 10.1152/ajpgi.00420.2002.—Gastrointestinal carcinoid cells secrete multiple neuroendocrine markers and hormones including 5-HT and chromogranin A. The intracellular signaling pathways that regulate production of bioactive molecules are not completely understood. Our aim was to determine whether activation of the ras/raf-1 signal transduction pathway in carcinoid cells could modulate production of neuroendocrine markers and hormones. Human pancreatic carcinoid cells (BON) were stably transduced with an estrogen-inducible raf-1 construct creating BON-raf cells. Activation of raf-1 in BON-raf cells led to a marked induction of phosphorylated MEK and ERK1/2 within 48 h. Importantly, raf-1 activation resulted in morphological changes accompanied by a marked decrease in neuroendocrine secretory granules by electron microscopy. Moreover, induction of raf-1 in BON-raf cells led to significant reductions in 5-HT, chromogranin A, and synaptophysin levels. Furthermore, treatment of BON-raf cells with MEK inhibitors PD-98059 and U-0126 blocked raf-1-mediated morphological changes and hormone suppression but not ERK1/2 phosphorylation. These results show that raf-1 induction suppresses neuroendocrine marker and hormone production in human gastrointestinal carcinoid cells via a pathway dependent on MEK activation.

MAP kinase; signal transduction; neuroendocrine tumors

GASTROINTESTINAL (GI) carcinoids are rare tumors that arise from the diffuse neuroendocrine system of the gut with a reported incidence of 1–8 per 100,000 (21, 23). Although GI carcinoids tend to be indolent, they frequently metastasize to the liver and are second only to colorectal carcinoma as the most common source of isolated liver metastases (11, 22). Hepatic resection remains the only curative treatment for patients with carcinoid liver metastases; however, most patients are unresectable at the time of diagnosis (11). Furthermore, patients with hepatic metastases frequently have debilitating symptoms, such as abdominal pain, flushing, bronchoconstriction, and diarrhea, due to the release of many bioactive hormones by the carcinoid tumor (15). Although the standard palliative treatment for these hormone-induced symptoms has been somatostatin analogs, such as octreotide, many patients become refractory and thus have limited options for palliation (22).

Growth of carcinoid and other neuroendocrine tumors has been shown to be dependent on growth factors and various downstream signaling pathways (33, 35). Therefore, we hypothesized that manipulation of certain cellular signaling pathways could potentially alter hormone secretion and growth of carcinoid tumors (33). It has been shown that the activation of the ras/raf-1 signal transduction pathway in other neuroendocrine tumors leads to a differentiation response (7, 10, 27). However, the biological consequences of raf-1 induction in GI neuroendocrine tumors, such as carcinoids, are unknown.

To study the effects of raf-1 activation in GI carcinoid cells in vitro, we used an established pancreatic carcinoid cell line, BON, derived from a metastasis of a human pancreatic carcinoid tumor (16, 32). BON cells synthesize and secrete several bioactive molecules including 5-HT (serotonin), chromogranin A, synaptophysin, neurotensin, and neuron-specific enolase (26, 38). Several studies have previously shown that hormone and peptide secretion by BON cells can be altered by treatment with IGF-1, phorbol esters, and δ-glucosamine, and even by mechanical stimulation (19, 20, 33).

In the present study, we show that BON cells have minimal levels of phosphorylated MEK and MAP kinases ERK1/2 at baseline. Activation of raf-1 in these cells led to high levels of phosphorylated MEK and ERK1/2 within 48 h. Induction of raf-1 and phosphorylated MEK and ERK1/2 in BON cells resulted in morphological changes accompanied by a marked decrease in the number of neuroendocrine secretory granules by electron microscopy. Raf-1 activation also caused significant reductions in the levels of 5-HT, chromogranin A, and synaptophysin. Importantly, although neuroendocrine marker and hormone levels were significantly reduced, raf-1 activation did not
stimulate proliferation of BON cells. Furthermore, by using MEK inhibitors, we demonstrate that these raf-1-mediated changes are dependent on MEK activation.

**MATERIALS AND METHODS**

**Cell culture.** Human pancreatic carcinoid cells (BON) were obtained from Drs. Mark Evers and Courtney Townsend, Jr. (University of Texas, Galveston, TX) and maintained in DMEM-nutrient mixture Ham's F-12K (DMEM-F-12K; 1:1; Gibco, Grand Island, NY) supplemented with 10% FCS (GIBCO, Grand Island, NY) and maintained in a humidified atmosphere of 5% CO2 at 37°C (3). Cell culture conditions for H727 cells and the packaging cell line PA317 have been previously described (9, 12). We have recently reported the creation of BON-raf cells (31).

**BON-raf cell line.** BON cells were stably transduced with the retroviral vector pLNC/raf-1:ER (31). This construct is an estrogen receptor-raf-1 fusion molecule, and contains the ligand-binding domain of the estrogen receptor fused to the raf kinase domain of c-raf-1 (6, 34). Derivation and maintenance of this cell line has previously been described (31). To induce raf-1 activity in BON-raf cells, 1 μM β-estradiol was added to the media. An equivalent dilution of ethanol, the carrier for the β-estradiol, was added to the α-medium. Equal dilution of ethanol, the carrier for the β-estradiol, was used to treat control cells. For the MEK inhibitor studies, cells were pretreated with 25–50 μM PD-98059 (7) and/or 5–10 μM U-0126 (17) or control (DMSO) for 45 min before the addition of β-estradiol or control carrier.

**Electron microscopy.** BON and BON-raf cells were grown to 50–75% confluency on 25 cm2 tissue culture dishes and then treated with control or estradiol for 48 h. Cells were then fixed with 3% glutaraldehyde in 0.1 M cacodylated buffer, dehydrated with varying concentrations of ethanol and 2-hydroxypropyl methacrylate, and imbedded in Eponate. For quantitation of neuroendocrine secretory granules, random images at similar magnitudes from each treatment group were selected. The number of secretory granules per cell was counted in a blinded manner by three independent observers.

**Western blot analysis.** Cells were trypsinized and cellular pellets were lysed in sample buffer (50 mM Tris, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% Na-deoxycholate, and 0.6 mM PMSF). Total cellular protein concentrations were determined with bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Cellular extracts (15 μg) were boiled with equal amounts (1:1) of loading dye (2% SDS, 20% glycerol, 0.1 M TRIS, 5% β-mercaptoethanol, and 0.04% bromphenol blue) for 10 min and through 10% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) by electroblotting. Membranes were blocked for at least 1 h in a milk solution (1× PBS, 5% dry milk, 0.05% Tween-20). The following primary antibody dilutions were used: ERK1/2 and phospho-ERK1/2 (1:1,000; Cell Signal Technology, Beverly, MA); MEK and phospho-MEK (1:1,000; Cell Signal Technology); chromogranin A (1:1,000; Zymed Laboratories, San Francisco, CA); and synaptophysin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibody incubations were performed either overnight at 4°C or for 1 to 2 h at room temperature. After primary antibody incubation, membranes were washed 1× 10 and 2× 5 min in PBS-T wash buffer (1× PBS, 0.05% Tween 20). Membranes were incubated with a 1:1,000 dilution of goat anti-rabbit secondary antibody (Cell Signal Technology) for 1 h, except for synaptophysin in which a dilution of goat anti-mouse antibody (1:1,000; Pierce) was used. Membranes were washed 1× 10 and 2× 5 min in PBS-T wash buffer and developed by enhanced chemiluminescence (Amerham, Arlington Heights, IL) according to the manufacturer's directions, except for synaptophysin, in which the Super West Pico chemiluminesence substrate (Pierce) was used.

**5-HT assay.** To determine serotonin levels in cellular extracts, we used a serotonin ELISA kit (Research Diagnostics, Flanders, NJ) per the manufacturer's instructions. 5-HT values were standardized for total protein content and quantitated relative to control cells. Samples from two independent experiments in triplicate were analyzed.

**Northern blot analysis.** Cells were trypsinized and total RNA was extracted by using the RNeasy Mini Prep Kit (Qiagen, Valencia, CA). RNA samples were then quantified and run on a formaldehyde-containing agarose gel. Passive transfer to a nylon membrane was performed by using the Northern Max protocol (Ambion, Austin, TX). Nonisotopically labeled chromogranin A and synaptophysin DNA probes were created by using the BrightStar psoralen-biotin labeling kit (Ambion). The blot was then hybridized with the DNA probe overnight at 43.5°C. The blot was washed and blocked per the Northern Max protocol and developed by using Ambion’s BrightStar BioDetect Kit.

The Northern blot was stripped by using boiling 0.1% SDS and then reprobed with a psoralen-biotin labeled RNA probe

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**Fig. 1. Western blot analysis for signal transduction proteins.** BON and BON-raf cells were treated with control (C) or estradiol (E2) and total cellular protein harvested as described in MATERIALS AND METHODS. A: induction of raf-1 leads to phosphorylation of ERK1/2 in BON-raf cells within 6 h. ERK1/2 remains phosphorylated at 24 and 48 h. Note the lack of phosphorlylated ERK1/2 in BON and untreated BON-raf cells. GADPH (G3DPH) was used to control for loading. B: induction of raf-1 leads to phosphorylation of MEK in BON-raf cells in a time frame similar to ERK1/2 phosphorylation (48 h shown). Note the minimal amount of phosphorylated MEK in BON and untreated BON-raf cells, whereas a baseline level of nonactive, unphosphorylated MEK was seen in all cells.
for GAPDH. The same protocol as above was performed with the exception of the hybridization temperature increased to 68°C.

**MEK in vitro kinase assay.** Cells were trypsinized and cellular pellets were lysed in sample buffer (50 mM Tris, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% Na/deoxycholate, and 0.6 mM PMSF). Total cellular protein concentrations were determined by BCA assay (Pierce). Cellular proteins (200 μg) were diluted with sample buffer to a concentration of 1 mg/ml. The protein samples were incubated with 5 μl of anti-MEK antibody (Cell Signal Technology) for 2 h at 4°C on an end-over-end rotator. We then added 50 μl of protein A agarose (Sigma) and rotated the mix overnight at 4°C. The samples were centrifuged at 4°C for 5 min at 14,000 rpm. The immunoprecipitates were washed twice with ice-cold 1× lysis buffer (Cell Signal Technology) with 1 mM PMSF and twice with 1× kinase buffer (Cell Signal Technology). Samples were centrifuged for 2 min at 4°C at 14,000 rpm between each wash. Immunoprecipitated products were incubated for 30 min at 30°C with 200 μM ATP and 2 μg of inactive glutathione S-transferase (GST)-p42 MAPK (Upstate Cell Signaling) in 1× kinase buffer. An aliquot of this reaction (20 μl) was then incubated with 2 μg of nonphosphorylated Elk-1 fusion protein (Cell Signal Technology) and 200 μM ATP for 30 min at 30°C. A single reaction without ATP served as a measure of background kinase activity. We added 20 μl of 4× SDS sample dye to each sample. Samples were then boiled for 5 min and centrifuged for 2 min. Final products were electrophoresed through 10% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membranes by electroblotting. Membranes were washed and blocked according to our Western blot protocol and then exposed to anti-Elk-1 antibody (1:1,000; Cell Signal Technology) in a 5% BSA solution (1× PBS, 0.1% Tween-20, 5% BSA) and dilution of goat anti-rabbit secondary antibody (1:1,000; Cell Signal Technology) in milk solution for 1.5 h. Membranes were washed 1×

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**Fig. 2.** Electron microscopy. BON and BON-raf cells after treatment with control or estradiol for 48 h. A: BON cells. B: BON-raf cells. Although numerous secretory granules are present in BON and untreated BON-raf cells (arrows), they are rarely seen in BON-raf cells treated with estradiol.
10 and 2 × 5 min in PBS-T wash buffer and developed by enhanced chemiluminescence (Amersham) per the manufacturer’s directions.

Growth assays. The methylthiazol tetrazolium (MTT) assay (Sigma) was performed. Briefly, BON and BON-raf cells were seeded in triplicate onto 24-well plates in phenol red-free DMEM/F-12K with 10% FCS at 3 × 10^4 for 24 h. Cells were then treated with estradiol or control. At 2-day intervals, medium was removed and replaced with a 250-μl medium containing MTT (0.5 mg/ml) and incubated at 37°C for 2 h. DMSO (750 μl; Sigma) was then added to each well, and absorbance at 540 nm was measured.

RESULTS

Raf-1 activation leads to increasing levels of phosphorylated MEK and ERK1/2. BON cells were stably transfected with an estradiol-inducible raf-1 construct, creating BON-raf cells (31). BON-raf cells do not differ from parental BON cells in phenotype (31). To determine the baseline levels of raf-1/MEK/MAP kinase pathway proteins in BON and BON-raf cells, we used Western analysis with antibodies against nonphosphorylated and phosphorylated MEK and ERK1/2, the factors downstream of raf-1. Figure 1 shows that inactive, nonphosphorylated MEK and ERK1/2 were present at high levels, whereas minimal or no detectable levels of active, phosphorylated MEK and ERK1/2 in BON and BON-raf cells at baseline. However, treatment of BON-raf cells with estradiol resulted in significant raf-1 activation manifested by a marked increase in the levels of phosphorylated ERK1/2 within 24 h, which persisted after 48 h (Fig. 1A). High levels of phosphorylated MEK were also seen in estradiol-treated BON-raf cells (Fig. 1B). Therefore, the addition of estradiol to BON-raf cells led to activation of the components of the raf-1/MEK/ERK1/2 signaling pathway. Similar levels of raf-1, MEK, and ERK1/2 activation were also achieved in BON-raf cells with the addition of 4-hydroxytamoxifen (1 μM), another estrogen receptor antagonist (data not shown).

Changes in morphology and neuroendocrine secretory granules. We have recently shown that induction of raf-1 in BON carcinoid cells results in striking morphological changes (31). BON-raf cells treated with estradiol are flatter and have sharper cellular borders and more cytoplasmic extensions under light microscopy (31). These morphology changes were visible in some cells as early as 12 h but appeared to affect all cells by 48 h. Although these changes resembled a morphological differentiation, examination under electron microscopy (EM) suggested otherwise. Neuroendocrine tumors such as GI carcinoids are characterized by the presence of numerous neuroendocrine secretory granules that can be seen on EM. BON cells treated with control or estradiol and BON-raf cells treated with control have abundant neuroendocrine secretory granules under EM (Fig. 2). In looking at numerous fields, rarely did any individual BON cell lack secretory granules. Significantly, induction of raf-1 by estradiol-treatment of BON-raf cells resulted in a 10-fold reduction in the number of neuroendocrine secretory granules at 48 h (P = 0.012) (Fig. 3).

Fig. 3. Presence of neuroendocrine secretory granules. The number of neuroendocrine secretory granules per cell was determined under electron microscopy in a blinded manner. Activation of raf-1 in BON-raf cells led to a 10-fold reduction in secretory granules, which was statistically significant (P = 0.012) compared with the other treatment groups.

Effects of raf-1 induction on neuroendocrine hormone levels. To determine whether the reduction in neuroendocrine secretory granules translated into lower levels of hormone, we carried out both Western analysis and ELISA. BON cells normally produce high levels of 5-HT, chromogranin A, and synaptophysin (26, 38). As shown in Figs. 4 and 5, BON and BON-raf cells both express high levels of these molecules. Although estradiol treatment did not alter the levels of neuroendocrine markers in BON cells, raf-1 activation through estradiol treatment in BON-raf cells led to a significant decrease in the levels of chromogranin A and synaptophysin (Fig. 4). Furthermore, induction of raf-1 in BON-raf cells resulted in a significant reduction in 5-HT levels by ELISA (Fig. 5). These reductions in neuroendocrine markers and hormone levels were detected as early as 24 h after raf-1 induction.

To determine whether the raf-1-induced reduction in neuroendocrine markers were the result of changes at the mRNA level, we performed Northern analysis on BON and BON-raf cells for chromogranin A and synaptophysin. As shown in Fig. 6, there were no significant differences in the levels of chromogranin A and synaptophysin messages in the cells before and after raf-1 activation. Thus raf-1-mediated reduction in neuroendocrine marker proteins and hormones does not appear to be due to changes at the mRNA level.

Raf-1-mediated hormone suppression and morphological changes are dependent on MEK activation. To determine whether the raf-1-induced effects were dependent on MEK activation, we used the well-characterized MEK inhibitors PD-98059 and U-0126 (17). As demonstrated above, induction of raf-1 in BON-raf cells leads to a significant reduction in 5-HT levels (Fig. 7). However, treatment of these cells with either MEK inhibitor, alone or in combination, blocked raf-1-in-
duced 5-HT suppression (Fig. 7). Similarly, the MEK inhibitors completely inhibited raf-1-mediated reduction in chromogranin A and synaptophysin protein levels (data not shown). Furthermore, treatment with PD-98059 and/or U-0126 blocked the BON-raf cellular morphology changes associated with raf-1 activation (Fig. 8). This blockade of raf-1 mediated hormone reduction and morphology changes by the MEK inhibitors was detectable within 12 h but much more noticeable by 48 h when the effects of raf-1 are more prominent. Of note, these inhibitors had no effect on native BON cells (data not shown).

Interestingly, although both MEK inhibitors blocked raf-1-mediated hormone suppression and morphology changes, treatment of BON-raf cells with PD-98059 and U-0126, alone or in combination, did not inhibit phosphorylation of ERK1/2. As shown in Fig. 9, the levels of phosphorylated ERK1/2 were similar in estradiol-treated BON-raf cells in the absence or presence of the MEK inhibitors. Furthermore, because ERK1/2 phosphorylation persists after blocking the raf-1-induced effects by treatment with the MEK inhibitors, phosphorylation of ERK1/2 alone does not appear to be sufficient to induce BON cell morphology changes and hormone reduction. In addition, these results also suggest that raf-1 can activate ERK1/2 through a MEK-independent pathway in BON carcinoid cells.

Because of the persistence of ERK1/2 phosphorylation, we planned to definitively prove that the MEK inhibitors blocked the raf-1-mediated morphology changes and hormone suppression in BON cells through direct inhibition of MEK, as opposed to nonspecific effects. As shown in Fig. 9, BON-raf cells pretreated with the MEK inhibitors for 45 min, followed

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Fig. 4. Western analysis for neuroendocrine markers. BON and BON-raf cells after treatment with control or estradiol. Western blots were performed on cellular extracts by using antibodies against chromogranin A (A) and synaptophysin (B). Levels of these hormones were standardized to G3PDH by image quantification software. Although BON and untreated BON-raf cells have high levels of these neuroendocrine hormones, there is a significant decrease in chromogranin A levels (reduction of 31% at 2 days, 68% at 4 days, and 50% at 6 days) and synaptophysin levels (reduction of 64% at 2 days and 29% at 4 days) after raf-1 activation in BON-raf cells.
by treatment with estradiol showed evidence of persistent ERK1/2 phosphorylation at 48 h. We then performed a MEK in vitro kinase assay on the same cellular extracts used for the Western blotting to determine whether MEK was indeed inhibited at 48 h. Immunoprecipitated MEK was used to phosphorylate an inactive GST-MAPK fusion protein, which, when activated, was able to phosphorylate Elk-1. As shown in Fig. 10, after 48 h of estradiol treatment and in the absence of MEK inhibitors, there was an increase in phosphorylation of Elk-1 due to MEK activity (lane 1). However, pretreatment with the MEK inhibitors followed by estradiol addition, markedly reduced the ability of immunoprecipitated MEK to generate phosphorylated Elk-1 in a coupled in vitro kinase assay (lanes 2 and 3). Thus these results show that ERK1/2 phosphorylation persists in estradiol-treated BON-raf cells despite adequate inhibition of MEK activity by MEK inhibitors. Therefore, raf-1 appears to be able to activate ERK1/2 through a MEK-independent pathway in BON carcinoid cells.

Effect of Raf-1 induction on BON cell proliferation. Activation of ras and/or raf-1 has been shown to promote cellular growth in a variety of tumors (14). To determine whether raf-1 activation in BON cells affects cellular proliferation, we used MTT assays. As shown in Fig. 11, there were no differences in proliferation rates of BON or BON-raf cells treated with control or estradiol over a 10-day period before reaching confluence. Similar results were obtained by cell counts with Trypan blue exclusion (data not shown).

DISCUSSION

The ras/raf-1 signal transduction pathway has been shown to play a significant role in the development of nonneuroendocrine tumors. Ras activating mutations are quite common, occurring in 85% of pancreatic and 50% of colonic adenocarcinomas (1, 5). In contrast, neuroendocrine tumors rarely have detectable ras mutations (25, 36, 37). No ras mutations have been found in an analysis of over 100 GI neuroendocrine tumors.
(carcinoids, insulinomas, gastrinomas, and glucagonomas) (25, 36, 37). Furthermore, our present data, as well as other recent studies of neuroendocrine tumor cell lines, suggest a different role for ras/raf-1 in neuroendocrine tumors. Activation of raf-1 has been shown to result in growth inhibition, phenotypic differentiation, and downregulation of the RET protooncogene in medullary thyroid cancer cells (8, 10). In small cell lung cancer cells, Ravi et al. (28) showed that raf-1 activation resulted in growth suppression, loss of soft agar cloning ability, and cell cycle arrest. In the present study, we found that activation of raf-1 in human BON carcinoid cells led to high levels of phosphorylated MEK and ERK1/2 within 48 h. Induction of raf-1 and phosphorylated MEK and ERK1/2 resulted in a striking morphological change resembling differentiation (31). Surprisingly, there was an 10-fold reduction in the number of neuroendocrine secretory granules seen by electron microscopy after raf-1 induction. Raf-1 activation also caused significant reductions in the levels of 5-HT, chromogranin A, and synaptophysin. It is possible that raf-1 activation in BON cells leads to a more undifferentiated phenotype, resembling progenitor neuroendocrine stem cells. Interestingly, others have demonstrated that neuroendocrine cells have the capacity to transdifferentiate. Schmied et al. (30) have shown that human islet cells can transdifferentiate into an undifferentiated phenotype. Importantly, although neuroendocrine marker and hormone levels were significantly reduced, raf-1 activation did not stimulate proliferation of BON cells.

Treatment of the BON-raf cells with MEK inhibitors PD-98059 and U-0126 led to two important observations. First, raf-1-mediated morphological changes and hormone suppression are dependent on MEK activation. This finding seems predictable given that MEK is the best characterized substrate for raf-1. However, similar high levels of activated ERK1/2 with MEK inhibition were also seen in BON-raf cells at other time points. A baseline level of nonactive, unphosphorylated ERK1/2 was seen in all cells.

**Fig. 8.** Raf-1-induced morphological changes are dependent on MEK activation. BON-raf cells were incubated with 50 μM PD-98059 and then treated with control or estradiol. In the absence of the MEK inhibitor, estradiol-treated BON-raf cells underwent dramatic morphological changes (top, right). Treatment with the MEK inhibitor blocked raf-1-induced morphological changes in the estradiol-treated BON-raf cells (bottom, right). Similarly, treatment of BON-raf cells with 10 μM U-0126 also resulted in reversal of phenotypic changes (data not shown).

**Fig. 9.** Western blotting for signal transduction proteins after treatment with MEK inhibitors. BON-raf cells were incubated with 50 μM PD-98059 and/or 10 μM U-0126 and then treated with control or estradiol for 48 h. Induction of raf-1 leads to phosphorylation of ERK1/2 in BON-raf cells. Notably, treatment with either MEK inhibitor, alone or in combination, failed to block ERK1/2 phosphorylation in estradiol-treated BON-raf cells. In fact, there does not appear to be any significant reduction in the levels of activated ERK1/2 with MEK inhibition. Similar high levels of activated ERK1/2 with MEK inhibition were also seen in BON-raf cells at other time points. A baseline level of nonactive, unphosphorylated ERK1/2 was seen in all cells.
several studies (2, 13, 24, 29) have recently observed raft-1-mediated effects in the absence of MEK activation through induction of other target signaling factors including MEK kinase 1, cell cycle proteins such as retinoblastoma protein, p53, cdc25, and apoptosis signal-regulated kinase 1. Moreover, studies using raft-1 knockouts have shown that MEK kinase activity is not necessary for raft-1 function (18).

Second, because ERK1/2 phosphorylation persists after blocking the raft-1-induced effects by treatment with the MEK inhibitors, phosphorylation of ERK1/2 alone does not appear to be sufficient to induce BON cell morphology changes and hormone reduction. Thus the morphological changes and hormone suppression seen in BON cells may be due to raft-1/MEK signaling through an alternative downstream pathway other than ERK1/2. In addition, the persistence of ERK1/2 phosphorylation also suggests that raft-1 can activate ERK1/2 through a MEK-independent pathway in BON carcinoid cells. This observation is further supported by the MEK in vitro kinase assay showing that the persistence of ERK1/2 phosphorylation occurs in the setting of adequate MEK inhibition by the MEK inhibitors. Although these findings are unique, other reports have illustrated ERK1/2 activation by raft-1 through MEK-independent pathways. Interestingly, Navas et al. (24) recently reported that receptor interacting protein-2 (RIP2) is involved in caspase activation and tumor necrosis factor receptor and Fas signaling and that it can be induced by raft-1 and can phosphorylate ERK1/2. Therefore, in the tumor necrosis factor signaling pathway, RIP2 takes the place of MEK to couple raft-1 to ERK1/2.

Our findings are especially interesting given the fact that other recent studies have focused on understanding the mechanisms that regulate hormone production in BON cells.

Kim et al. (20) showed that mechanical stimulation of BON cells leads to increased levels of 5-HT secretion. They determined that the mechanism of this enhanced hormone release was stimulation of a G protein-coupled receptor leading to mobilization of intracellular calcium (20). The same group also discovered that exposure of BON cells to high concentrations of d-glucose activates other signaling pathways to increase levels of 5-HT secretion (19). Furthermore, Zhang et al. (38) showed that phorbol ester treatment of BON cells results in a persistent release and cellular depletion of chromogranin A. They further demonstrated that these effects were mediated through the PKC pathway (38). Although raft-1 activation also led to marked cellular depletion of chromogranin A and other hormones, we did not see enhanced secretion of hormones as seen with phorbol ester treatment. In fact, when testing BON-raft cellular supernatants for 5-HT after raft-1 activation, we saw slightly diminished levels of 5-HT.

Although our present study is the first to examine the role of raft-1 in GI carcinoid cells, others have looked at pathways associated with raft-1. Bold et al. (4) studied the effects of NGF and its associated receptor trkA. They demonstrated that NGF acts as a mitogen for BON cells without any effect on cellular phenotype or hormone production (4). Interestingly, they hypothesized that trkA could be signaling through raft-1 and MAP kinases. However, our findings showed that activation of raft-1 led not only to changes in cellular morphology, but also to the reduction in hormone production. These differences could be due to the intensity of the raft-1 signals generated by overexpression of raft-1 versus a ligand-receptor interaction. It is also possible that trkA could be signaling through parallel pathways, such as phosphatidylinositol 3-kinase, and that BON cell growth and differentiation are controlled by a combination of these signaling pathways.

In conclusion, we have demonstrated that overactivation of raft-1 suppresses hormone production by human carcinoid tumor cells. Further characterization of this pathway will determine whether modulation of the raft-1 signaling could play a potential role in the management of patients with carcinoid tumors. Furthermore, these findings may permit development of components of the raft-1 pathway as therapeutic targets in the treatment and palliation of GI neuroendocrine tumors.
We thank Dr. Norman Drinkwater for his significant contributions to the experimental design and support, and review of the manuscript. We also thank Drs. Eric Nakakura, Barry Nelkin, and Douglas Ball for their helpful comments, Drs. Mark Evers and Courtney Townsend, Jr. for the BON cell line, and Dr. Martin McMahon for the inducible ras-1 vectors.

DISCLOSURES

This study was supported, in part, by American Cancer Society Grant IRG-58-011-44-04 (to H. Chen), James Ewing Oncology Fellowship Award for Basic Research from the Society of Surgical Oncology (to H. Chen), American Surgical Association Foundation Fellowship Award (to H. Chen), a grant to the University of Wisconsin Medical School under the Howard Hughes Medical Institute Research Resources Program for Medical Schools (to H. Chen), and National Cancer Institute Grant T32-CA-90217 (to R. S. Sippel).

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