Constitutive ERK1/2 Activation in Esophagogastric Rib Bone Marrow Micrometastatic Cells Is MEK-independent*

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In this study, we examined the mitogen-activated protein kinase (MAPK) cascade in micrometastatic cell lines generated from rib bone marrow (RBM) of patients undergoing resection of esophagogastric malignancies. The molecular mechanism(s) involved in esophagogastric MAPK activation have not previously been investigated. Constitutive activation of both ERK1 and -2 isoforms was evident in each of the five RBM cell lines. Elk-1, a transcription factor activated by the ERK1/2 pathway was also found to be constitutively activated. Cell lines generated from metastases of involved lymph nodes (OC2) and ascites (OC1) of patients with esophageal cancer do not display, however, hyperphosphorylation of ERK1/2. Constitutive RBM ERK1/2 activation is protein kinase C and phosphatidylinositol 3-kinase dependent. Surprisingly, constitutive ERK1/2 activation is MEK-independent. Pharmacological inhibition of MEK with two specific inhibitors, PD 98059 and U0126, were both ineffective in blocking ERK activation. Similarly, the use of a dominant negative MEK mutant was without effect. Interestingly, experiments overexpressing two different dominant negative Pak1 mutants significantly reduced RBM ERK1/2 activation, albeit not to the same extent for all cell lines. We also examined the role of three different phosphatases, PAC1, MKP-1, and -2. While RBM ERK1/2 activation was found to be PAC1- and MKP-2-independent, surprisingly, MKP-1 was down-regulated in all five RBM cell lines. In conclusion, we provide evidence for the first time for a MEK-independent constitutive ERK1/2 activation pathway in esophagogastric RBM cell lines. These findings have important implications for drug treatment strategies which currently target MEK in other forms of cancer.

Esophageal cancer is an aggressive tumor which responds poorly to treatment and has a poor prognosis (1, 2). Approximately half of patients diagnosed with localized esophageal cancer die of metastatic disease within the first 2 years following tumor resection. We have succeeded in developing a number of esophagogastric cell lines from the rib bone marrow of patients (3). We have previously shown that these cells are viable, proliferate, and grow independently in tissue culture and form malignant tumors in athymic nude mice (4). These metastatic cells are representative of the disseminated progenitors of secondary tumors and are the appropriate targets for treatment.

The precise molecular events leading to the acquisition of the metastatic phenotype remain largely unknown. Members of the Ras superfamily of small GTP-binding proteins have been implicated in tumor progression and are found to be activated in 20 to 30% of tumors (5). In its active GTP-bound state, Ras activates the serine threonine kinase Raf (6). Raf upon activation in turn phosphorylates the dual specific kinase MEK (also known as MAPK kinase or MAPKK) which in turn phosphorylates the MAP kinases ERK1 and -2 (for extracellular regulated kinases 1 and 2). Although MEK is the only known kinase directly downstream of Raf, there are several lines of evidence suggesting that Raf can also activate other effectors (7, 8). In vitro MEK1 is phosphorylated by ERKs, Cdk2, and the p21-activated kinase Pak1 (9, 10). The Pak kinases Pak1, -2, and -3, are a family of protein kinases that are regulated by GTP-bound Rac and Cdc42 and are candidates for effectors that mediate both actin and JNK signaling (for review, see Ref. 11).

The MAPKs are activated by the reversible dual threonine and tyrosine phosphorylation of a conserved T-X-Y motif (12). The reversible nature of MAPK phosphorylation suggests that phosphatases play a key role in regulating MAPK activity (for review, see Ref. 13). MAPK phosphatase-1 and -2 (MKP-1, -2) as well as PAC1, a phosphatase which shares a highly homologous C-terminal catalytic domain with MKP-1 (14), are known to inactivate ERKs (15) and possibly JNKs/stress-activated protein kinases (16, 17). While PAC1 is expressed predominantly in hematopoietic cells, MKP-1 and -2 are more widely expressed and are induced by growth factors and genotoxic and environmental stresses (17, 18).

Constitutive activation of ERKs in human malignancies has been previously documented (19–22), albeit the detailed mechanism(s) underlying such activation have not been well char-

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acertized. In this study, we undertook to characterize the MAPK signaling pathway in micrometastatic cell lines generated from rib bone marrow of patients undergoing resection of esophageogastric malignancies, which until now had remained unexplored. Using complementary genetic and pharmacological approaches, we now demonstrate that in all five esophageogastric rib bone marrow (RBM) cell lines examined, both ERK-1 and -2 isoforms are activated in a MEK- and Raf-1-independent manner. In contrast, however, ERK1/2 activation was found to be PKC-, PI3K- and Pak1-dependent. Moreover, we did not detect PAC1 expression, but found that MKP-1 but not MKP-2 was down-regulated. In addition, the activation of ERK1/2 correlated with increased activation of the downstream transcription factor Elk-1.

MATERIALS AND METHODS

Reagents

EDTA, EGTA, leupeptin, Nonidet P-40, soybean trypsin inhibitor, aprotinin, β-glycerophosphate, sodium orthovanadate, magnesium chloride, sodium fluoride, and myelin basic protein were purchased from Sigma. Cell culture medium, fetal calf serum (FCS) and antibiotics (penicillin, streptomycin, amphotericin B) were purchased from Life Technologies, Inc. (Dublin, Ireland). Phorbol 12-myristate 13-acetate (PMA), GF 109203X, LY294002, wortmannin, H-89 (N-[2-(p-chloroamino)-2-aminophenyl]-5-isooquinoline-sulfonamide) and the two MEK inhibitors, 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059) and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl)-amino-3H-89 (–bromocinnamylamino)ethyl]-5-isoquinolinesulfinamide), the p38 MAPK antibody kits were purchased from New England Biolabs (Cruz Biotechnology Inc., Santa Cruz, CA). Chemiluminescent substrates were used to reveal positive bands that were visualized after exposure to Hyperfilm ECL (Amersham Pharmacia Biotech). All immunoblots were performed in triplicate.

Cells

Generation of the esophageogastric metastatic rib bone marrow cell lines (lines 1–5) used throughout this study have previously been described in detail by our group, and were obtained from patients undergoing resection of esophageogastric cancers (4). Briefly, RBM1 and -5 cell lines are cells cultured from two different patients with adenocarcinoma of the gastroesophageal junction. RBM2 and -4 cell lines are cells cultured from two different patients with adenocarcinoma of the esophagus and finally, the RBM3 cell line are cells cultured from a patient with squamous carcinoma of the esophagus. OC1 and OC2 are squamous cell lines established from malignant ascites and a lymph node, respectively, of two patients with esophageal cancer. Finally, OCS are adenocarcinoma cells established from a metastatic lymph node of a Barrett’s esophageal lesion. We used normal human bone marrow stromal (HBMS) cells throughout this study as control. CACO-2, HT-29, and SW480 cells were also employed for comparative purposes in some experiments throughout this study.

Cell Culture

RBM1–5, OC1, -2, and -3, CACO-2, HT-29, and SW480 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mg/ml-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. Cells were washed twice with DMEM, and 1 x 10^5 cells/well were plated into 6-well tissue culture plates. After 48 h cells were cultured in medium supplemented with antibiotics and 0.5% FCS for 5 experiments performed unless otherwise stated. HBMS cells, obtained from the ATCC (Rockville, MD), were cultured in DMEM supplemented with 10% FCS, 2 mg/ml-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 1.5 μg/liter sodium bicarbonate, 4.5 μl-glucose, and 1 x 10^5 cells/well in DMEM supplemented with antibiotics and 0.5% FCS, for all experiments performed unless otherwise stated.

Plasmids

cDNA expression plasmids utilizing the cytomegalovirus promoter to express Myc-tagged Pak1, Pak1R299, Pak1L83,L86, and Pak1L83,L86,R299 were the generous gift of Dr. J. Field (University of Pennsylvania School of Medicine). The Pak constructs have been previously described in detail (24–26). Briefly, Pak1 is wild type Pak, Pak1R299 is a hyperactive Pak, Pak1L83,L86,R299 lacks kinase activity and Pak1L83,L86 is a mutant that lacks kinase activity and also fails to bind either Rac or Cdc42 (26). The dominant-negative form of MEK1 was a generous gift of Drs. O. A. Coso and J. S. Gutkind (National Institutes of Health).

Immunoblot Analysis

RBM1–5 cell lines, OC1, -2, and -3, CACO-2, HT-29, SW480, and HBMS cells were cultured in DMEM supplemented with antibiotics and 0.5% FCS. Cells were lysed in ice-cold lysis buffer as previously described (27). Supernatants were used for immunoblotting with antibodies for the phosphorylated or total p42/p44 MAPK, JNK1/2, or p38, using the experimental conditions described by the manufacturer (New England Biolabs). Similarly, membranes were incubated with antibodies for Raf-1 (C-12), and MKP-1 (M-18) and -2 (S-18) (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Chemiluminescent substrates were used to reveal positive bands that were visualized after exposure to Hyperfilm ECL (Amersham Pharmacia Biotech). All immunoblots were performed in triplicate.

In Vitro MAPK Assay

ERK, MEK, and Raf kinase assays were performed as previously described (28) with minor modifications. Briefly, cells were lysed in 100 μl of modified RIPA buffer containing 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 10 mM sodium fluoride, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 0.5% Nonidet P-40. Supernatants were used for immunoblotting with antibodies for Raf-1 (C-12), anti-MEK-1 (M-18), and -2 (S-18) (Santa Cruz Biotechnology Inc.), anti-MAPK (Santa Cruz Biotechnology, Inc.) antibodies preabsorbed to protein A-Sepharose beads (Amersham Pharmacia Biotech). Immunoprecipitates were washed three times with cold phosphate-buffered saline containing 1% Nonidet P-40 and 2 mM Na3VO4, once with 100 mM Tris, pH 7.5, 0.5% LiCl, and once with 1 x 10-5 M of phosphatase inhibitors (12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 mM vanadate) and resuspended in 30 μl of reaction buffer (kinase reaction buffer with 20 μM ATP, 1 μCi of [γ-32P]ATP, and 3.3 mM dithiothreitol and 0.5 μg of myelin basic protein, 0.5 μg of kinase inactive GST-ERK1/K71A (Upstate Biotechnology Inc.) or 0.1 μg of kinase inactive GST-MEK-1/K71A (Upstate Biotechnology, Inc.). Incubations were carried out for 30 min at 30 °C. Reactions were terminated by the addition of 10 μl of Laemmli sample buffer. Samples were run on a 10 or 7.5% SDS-PAGE gel, and visualized by autoradiography. The MAPK, MAPKK, and Raf-1 activity was defined as the amount of radioactive incorporation into myelin basic protein, GST-ERK1, or GST-MEK-1, respectively, under these conditions. Alternatively, the reaction was terminated by spotting 30 μl of reaction mixture onto 1-inch squares of phosphocellulose paper (Whatman International Ltd., Maidstone, United Kingdom). Free [γ-32P] was removed by three washes in 180 mM phosphoric acid as previously described (26). Incorporated radioactivity was determined by liquid scintillation counting. All assays were performed in triplicate.

Semi-quantitative RT-PCR

Total RNA from RBM1–5, OC1, -2, and -3, CACO-2, HT-29, SW480, and HBMS cells was isolated using the acid guanidium:phenol: chloroform method (29). Total RNA was digested with RNase-free DNase I (RQ1; Promega, Madison, WI) and subsequently re-extracted with the acid guanidium:phenol:chloroform method. PCR was performed in a final volume of 50 μl containing 2 μl of CDNA, 15 pmol of each primer, 1.5 μl MgCl2, 200 μM each dNTP, and 1 unit of Taq DNA polymerase, all in 1 x supplied Taq buffer (Promega). Sequences of primers used were: β-actin (primer positions correspond to nucleotides 541–571) (sense primer) and nucleotides 1171–1201 (antisense primer) on human β-actin mRNA, sense: 5′-TGACGGGTTGACCCACACTGTTG- GCCCATCTA-3′; β-actin, antisense: 5′-CTAGAAAGCTTGGGCGT- GACAGGTAGGAGG-3′; PAC-1 (20), sense: 5′-TTGCCCTACCTTGCTTGTG- GGG-3′; PAC-1, antisense: 5′-GTCCTAATGCTGAGCAGTG-3′. The PCR conditions were as follows: 94 °C, 15 s; 64 °C, 20 s; 72 °C, 1 min, following an 80 °C, 5-min step prior to the addition of Taq (hot start),
with 23 cycles for β-actin and 33 cycles for PAC-1. 10 μl of each PCR was resolved on ethidium bromide-stained 3:1 NuSieve:Agarose gels.

**Transgenic Transfection of Cells**

To investigate constitutive Elk-1 transcriptional activation, we employed the PathDetect trans-reporting system as previously described but with minor modifications (27). Plasmid DNAs were transiently transfected into RBM(1–5), OC1, -2, and -3 using LipofectAMINE™ 2000 Reagent (Life Technologies) according to the manufacturer’s instructions. For all experiments, 1 μg of Renilla luciferase vector pRLSV40 (Promega Corp.) was included which provides constitutive luciferase expression in transfected cells and serves as an internal control to normalize transfection efficiency. Cells were co-transfected with luciferase reporter (pFR-Luc) and Renilla plasmids, along with the GAL-4-c-Elk-1 expression plasmid or pPC-dbdl as a negative control. Transfections were carried out using a total of 5 μg of DNA (3 μg of GAL-4-c-Elk-1 plus 1 μg of pFR-Luc plus 1 μg of Renilla or 3 μg of pPC-dbdl plus 1 μg of pFR-Luc plus 1 μg of Renilla). Forty-eight hours following transfection, cells were washed twice in phosphate-buffered saline and refed with DMEM supplemented with antibiotics and 0.5% FCS for a further 48 h. Both firefly- and Renilla-luciferase activities were measured using a commercial Dual Luciferase™ Reporter Assay Kit (Promega) following the manufacturer’s instructions.

**Cell Proliferation Assays**

**MTT Assay—**RBM(1–5) cell lines were seeded onto 96-well plates at 5 × 103 cells per well in growth medium and grown overnight prior to the initiation of any experimental treatments. Following the indicated treatments, MTT (5 μg/ml) was added to each well of a 96-well plate and the reduction of MTT was assayed to calculate cell numbers as previously described (30).

**[3H]Thymidine Incorporation—**Cells were seeded sparsely in 6-well plates, and grown in DMEM supplemented with 10% FCS and antibiotics for 24 h. The cells were then starved by culturing in the same medium without serum for 48 h. The medium was then removed, and cells were refed with medium containing 10% FCS and 0.5 μCi/well [3H]thymidine (Amersham Pharmacia Biotech). The cells were incubated for 48 h after which [3H]thymidine incorporation was performed as previously described (31).

**Cytotoxicity Assay—**For experiments using various kinase inhibitors, cytotoxicity was measured by the MTT cell viability assay described above.

**Statistical Analysis**

Results are expressed as mean ± S.E. Statistical comparisons were made by using analysis of variance with subsequent application of Student’s t test, as appropriate.

**RESULTS**

**Constitutive Activation of ERK1/2 in RBM(1–5)—**To determine whether ERKs are activated in esophagogastric RBM we evaluated the phosphorylation of ERK1/2 using phosphospecific antibodies as an index of ERK1/2 activation (27). All five of the RBM cell lines when cultured in 0.5% FCS, had substantially higher levels of phosphorylated ERK1/2 when compared...
Constitutive ERK1/2 Activation in Esophagogastric RBM

Fig. 2. Effect of different inhibitors on constitutive p44/p42 MAPK activation in esophagogastric micrometastatic rib bone marrow cell lines. Immunoblot analysis (A and B) and immunoprecipitation (C) were carried out as described under “Materials and Methods.” HBMS cells, OC1, -2, and -3 and RBM1 were grown in DMEM supplemented with antibiotics and 0.5% FCS (A and C). HBMS cells were cultured with and without 10% FCS (S) or stimulated with PMA (100 nM) for 5 min (B). Cells were preincubated with GF 109203X (GF) (5 μM), PD 98059 (PD) (20 μM), U0126 (20 μM), LY294002 (LY) (20 μM), wortmannin (W) (100 nM), H-89 (10 μM), and staurosporine (St) (100 nM) for 1 h prior to analysis. Twenty μg of protein was loaded per lane (A-C). The figure is representative of four separate blots. C, results are expressed as mean ± S.E. from four independent experiments.

with HBMS cells, HT-29, CACO-2, SW480, and OC1, -2, and -3 (Fig. 1B). ERK1/2 was defined as constitutively activated in RBM(1–5) as cells grown in the presence of serum (S) or treated with PMA for 5 min did not demonstrate a further increase in phosphorylation (Fig. 1B). Results are shown for RBM1 but similar results were observed for RBM(2–5) (data not shown). Since previous reports have linked constitutive ERK activation with ERK hyperexpression (20, 22), we examined ERK expression by immunoblot analysis. We failed to see any significant difference in ERK protein expression when RBM(1–5) cell lines were compared with HBMS cells, HT-29, CACO-2, SW480 and OC1, -2, and -3 (Fig. 1A).

To verify that the observed phosphorylated ERK1/2 in RBM(1–5) was active and capable of in vitro phosphorylation of cellular proteins, we used immunoprecipitated ERK from equal volumes of protein extracted from RBM(1–5). HBMS cells and cells grown in the presence of 10% FCS were employed for comparison purposes in in vitro kinase assays with myelin basic protein as a substrate. A representative experiment is shown in Fig. 1C which corroborates Western blot data analysis (Fig. 1B). Again demonstrating a marked increase in the activation state of ERK in RBM cells compared with normal HBMS cells. No further increase in ERK activity was observed when RBM1 cells were grown in the presence of 10% FCS (Fig. 1C). Similar results were obtained for RBM(2–5) (data not shown).

To address the question of whether other members of the MAPK cascade could also be constitutively activated, we examined the phosphorylation of both JNK and p38. We failed to see either JNK or p38 phosphorylation in each of the five cell lines examined (Fig. 1, D and E). Treatment of the RBM1 cell line with UV for 5 min is shown as a positive control (Fig. 1, D and E).

PKC, and PI 3-Kinase but Not MEK Are Involved in RBM(1–5) ERK1/2 Activation—To address the mechanism(s) of constitutive ERK1/2 activation in RBM(1–5), cells were pretreated for 1 h with GF 109203X (5 μM), a PKC inhibitor (32), GF 109203X (5 μM) abolished ERK1/2 activation while the PKA inhibitor H-89 (10 μM) was without effect (Fig. 2A). Likewise, staurosporine (100 nM), a nonspecific serine threonine kinase inhibitor, also abolished RBM(1–5) ERK1/2 constitutive activation (Fig. 2A). Results are shown for RBM1 but similar results were observed for RBM(2–5) (data not shown). Since MEK is the upstream activator of ERKs (33, 34), we addressed the role of MEK1/2 in the observed constitutive activation of ERK-1 and -2 in RBM(1–5). Cells were incubated with PD 98059 (20 μM) or U0126 (20 μM), both inhibitors of MEK (35, 36). The compound U0126 has been identified as a selective inhibitor of MEK-1 and MEK-2, both in vitro and in vivo (36). Surprisingly, we observed that both inhibitors failed to alter the phosphorylation of ERK1/2 in RBM (1–5) (Fig. 2A). Again, results are shown for RBM1 but similar results were observed for RBM(2–5) (data not shown). To verify that both inhibitors, however, could abolish ERK phosphorylation, HBMS cells were pretreated with PD 98059 (20 μM) and U0126 (20 μM) for 1 h. ERK1/2 phosphorylation was completely abolished in the presence of both inhibitors when serum-starved HBMS cells were cultured in DMEM plus 10% FCS (S) for 24 h or treated with PMA (100 nM) for 5 min (Fig. 2B). Therefore, both inhibitors are fully capable of “shutting down” activation of the MAPK pathway. To investigate the role that PI3K may have in RBM(1–5) constitutive ERK1/2 activation, cells were treated with two PI3K inhibitors LY294002 (20 μM) and wortmannin (100 nM) (37). Both inhibitors abolished the phosphorylation of ERK1/2 in RBM(1–5) (Fig. 2A). Similarly, ERK1/2 kinase activity could be altered pharmacologically. GF 109203X (5 μM), as well as LY294002 (20 μM) and wortmannin (100 nM) abolished ERK1/2 kinase activity (Fig. 2C), while PD 98059 (20 μM), U0126 (20 μM), and H89 (10 μM) were without effect (Fig. 2C).

Constitutive ERK1/2 Activation is Raf- and MEK-independent in RBM(1–5)—Since both PD98059 and U0126, two specific inhibitors of MEK (35, 36), failed to alter the activation of ERK1/2 in RBM(1–5) (Fig. 2, A and C), we turned our attention to MEK. We examined the expression, phosphorylation, and activation of MEK in RBM(1–5). The phosphospecific MEK1/2
antibodies (New England Biolabs) only recognize the phosphorylated forms of MEK1/2 but do not distinguish between the two isoforms. As expected, serum-starved HBMS cells, as well as OC1, did not exhibit MEK1/2 phosphorylation (Fig. 3C). Similar results were found for OC2, -3, CACO-2, HT-29, and SW480 (data not shown). However, MEK1/2 was not found to be phosphorylated in serum-starved RBM(1–5) (Fig. 3D), despite the existence of ERK activation. When RBM1, HBMS, and OC1 cells were cultured in DMEM supplemented with 10% FCS (S) or treated with PMA (100 nM) for 5 min, MEK1/2 was phosphorylated (Fig. 3, B and C). Results are shown for RBM1, but similar observations were noted for RBM(2–5) (data not shown). Since a previous report has linked MEK hyperactivation with ERK constitutive activation (21), we performed immunoblot analysis to determine the expression level of MEK as well as ERK.

Dominant Negative MEK Does Not Disrupt ERK1/2 Constitutive Activation in RBM(1–5)—To further confirm that ERK activation is MEK-independent in RBM(1–5), cells were stably transfected with a DN MEK mutant. In agreement with our results using the two specific MEK inhibitors, we failed to see any change in ERK1/2 phosphorylation in RBM(1–5) when cells were transfected, we analyzed the level of MEK protein in RBM(1–5) versus HBMS, OC1, -2, and -3 cells (Fig. 4A). Unlike previous reports (21), we failed to see any significant differences in MEK1/2 expression for all cells analyzed (Fig. 3A). To reconcile the absence of MEK activation in RBM(1–5) we also performed a MEK kinase assay using kinase inactive GST-ERK-1 (K71A, Upstate Biotechnology Inc.) as substrate (28). Consistent with our immunoblot results, we failed to observe an increase in MEK kinase activity (Fig. 3, D and E). Results are shown for RBM1, but were similar for RBM(2–5) (data not shown). Cells cultured in the presence of serum (S) or treatment with PMA (100 nM) for 5 min, however, showed a marked increase in MEK activity, as assessed by GST-MEK-1 phosphorylation (Fig. 3G) as previously described (38). Thus, RBM(1–5) have a functional Raf-1 and MEK as well as ERK.

Pak1 Contributes to the Constitutive Activation of ERK1/2 in
RBM(1–5)—Since both MEK inhibitors or DN MEK failed to alter the activation of ERK1/2 in RBM(1–5), we sought to identify other kinases which may lie upstream in the MAPK signaling pathway of these cells. Since we observed that the two PI3K inhibitors LY294002 and wortmannin both abolished ERK1/2 activation in RBM(1–5) (Fig. 2, A and C), we turned our attention to Pak, a kinase which has previously been shown to be activated by PI3K (25). Pak has been shown to activate JNK (39), as well as ERK (40). We developed stable RBM(1–5) cell lines that expressed the various Pak mutants, confirmed by Western blot analysis of the Myc-tagged plasmids (Fig. 5A). Pak1 is wild type Pak, Pak1R299 lacks kinase activity, Pak1L83,L86 lacks kinase activity, and Pak1L83,L86,R299 is a mutant that lacks kinase activity and also fails to bind either Rac or Cdc42, and Pak1L83,L86 is a hyperactive Pak. The two kinase-deficient Pak mutants are both dominant negative forms of Pak. Pak1 is seen as a 65-kDa band. Therefore, we tested the effects of the different Pak mutants versus empty vector on the constitutive activation of ERK1/2 in RBM(1–5). We observed that wild type Pak1 did not affect ERK1/2 activation (Fig. 5B). When we tested the two kinase mutants Pak1R299 and Pak1L83,L86,R299, however, we consistently found that both reduced ERK1/2 phosphorylation in all of the RBM cell lines, albeit not to the same extent. We observed that the two Pak1 mutants continuously had less of an effect on RBM4 and -5 ERK activation in comparison to RBM1–3 (Fig. 5, C and D). A reduction in phosphorylated ERK in cells overexpressing the dominant negative Pak mutants has previously been described by Tang et al. (26). Interestingly, cells overexpressing the hyperactive Pak1L83,L86 showed a further increase in ERK1/2 phosphorylation (Fig. 5, E and F). This increase in ERK1/2 activation was MEK-independent but PI3K dependent (Fig. 5, E and F). Again, results are shown for RBM1 but were similar for RBM(2–5) (data not shown). We also examined whether cells expressing the different Pak1 mutants could alter the levels of ERK and MEK protein expression. In all five cell lines expressing the four different Pak1 mutants, we failed to see any changes in protein expression (data not shown). Since Pak has been shown to activate JNK (39), we also examined whether the hyperactive Pak1 mutant, Pak1L83,L86,R299, which brought about a further increase in ERK activation, could increase JNK activation in RBM(1–5). We failed, however, to see up-regulation of JNK in RBM(1–5) (Fig. 5G).

RBM(1–5) Demonstrate Constitutive Transcriptional Activation of Elk-1—To further evaluate the level of activation in RBM(1–5), we investigated whether the observed upstream constitutive activation of ERK1/2 may influence gene expression. RBM(1–5) transiently transfected with GAL-c-Elk-1 demonstrated an elevated level of Elk-1 activation when compared with HBMS, OC1, -2, and -3 (Fig. 6A). To investigate the upstream kinases involved in the Elk-1 activation, cells were treated with the PKC inhibitor GF 109203X (5 μM, GF 109203X brought about a significant reduction in the activation of Elk-1 (Fig. 6B). Similarly, the PI3K inhibitors LY294002 (20 μM) and wortmannin (100 nM), reduced the levels of Elk-1 activation (Fig. 6B). In contrast, however, both PD 98059 (20 μM) and U0126 (20 μM), failed to abrogate the levels of Elk-1 activation, thereby implying a MEK-independent Elk-1 up-regulation in RBM(1–5) (Fig. 6B).

Since we observed that the two kinase-deficient Pak1 mutants, Pak1L83,L86,L86,R299 and Pak1L83,L86,L86,R299, were capable of reducing the levels of ERK1/2 activation in RBM(1–5) (Fig. 5, C and D), we decided to investigate if they could also alter the levels of constitutive Elk-1 activation. Stable cell lines overexpressing wild type Pak1 (Pak1) did not display altered levels of Elk-1 activation in all five RBM cell lines examined (Fig. 6C). Both kinase-deficient Pak mutants, Pak1L83,L86,L86,R299 and Pak1L83,L86,L86,R299 however, were efficacious in significantly reducing the levels of Elk-1 activation in RBM1 and -2 but not in RBM3, -4, and -5 (Fig. 6C). The hyperactive mutant, Pak1L83,L86,L86,R299, significantly increased the level of Elk-1 activation in RBM2 and -3 but not in RBM1, -4, and -5. Thus, while Pak1 seems to play a definitive role in ERK1/2 activation
in RBM(1–5), its role in Elk-1 activation is more complex. Pak1 appears to influence Elk-1 activation in RBM1, -2 and -3, but is ineffective in RBM4 and -5, suggesting that there may be other kinases operating in these two cell lines lying outside of the Pak pathway (39, 40) which influence the level of activation of Elk-1. These results are consistent with those for ERK activation where Pak1L83,L86,R299 and Pak1R299 were less effective in RBM4 and -5 ERK down-regulation, thereby indicating that these two cell lines may be differentially regulated compared with RBM1–3.

Constitutive Activation of ERK1/2 in RBM(1–5) Is MKP-1-dependent But MKP-2 and PAC1-independent—The constitutive activation of ERKs suggested the possibility of an abnormal dephosphorylation mechanism of ERKs. PAC1, a member of the ERK phosphatase family is induced in response to ERK activation (14, 41). Therefore, to examine whether abnormal ERK down-regulation mechanisms were present, we examined PAC1 gene expression by RT-PCR because PTPases, including PAC1, are principally up-regulated at the transcription level in response to ERK activation (42). PAC1 gene expression was not observed in RBM(1–5) or OC2 cells, but was present in OC1, -3, HT-29, CACO-2, SW480, and HBMS cells showing no ERK activation, and could be up-regulated in the presence of serum (Fig. 7A). We then examined the role of two other protein-tyrosine phosphatases MKP-1 and -2 using antibodies which are specific for both. Like PAC1, both MKP-1 and -2 are reported to be up-regulated upon ERK activation (16, 43). Interestingly, in our study, we found that the levels of MKP-1 expression in RBM(1–5) showing constitutive ERK1/2 activation was significantly lower than that in unstimulated HBMS, CACO-2, HT-29, SW480, OC1, -2, and -3 cells showing no ERK activation (Fig. 7B). No changes in MKP-2 expression were observed (Fig. 7C).

RBM(1–5) Cell Proliferation is PKC- and PI 3-Kinase-dependent But MEK-independent—The MAPK pathway is essential in cellular growth and differentiation (44). Thus, we examined the role of the constitutive activation of ERK1/2 on RBM(1–5) cell growth. To investigate the role of MEK in RBM cell proliferation, cells were treated with PD 98059 (20 μM). This MEK inhibitor failed to significantly reduce the cell number (using the MTT assay) or DNA synthesis (using [3H]thymidine incorporation) (Fig. 8). PD 98059 was effective, however, in significantly reducing HT-29 cell growth (data not shown). We also investigated the role of PKC, and PI3K in RBM(1–5) cell proliferation. PKC inhibition with GF 109203X (5 μM) or pretreatment with PMA (100 nM) for 48 h which down-regulates PKC (27) significantly reduced cell growth (Fig. 8). Similar results were observed when cells were treated with the PI3K inhibitors, LY294002 (20 μM) and wortmannin (100 nM). Wortmannin is unstable in solution and PI3K levels remain suppressed for only 9–12 h after drug addition (45), thus cells were washed and pretreated a second time with wortmannin (100 nM) for another 12 h. Thus, ERK1/2 constitutive activation in RBM(1–5) correlates with cellular proliferation.

**DISCUSSION**

The present results have identified for the first time that the mitogen-activated protein kinases ERK1 and -2 as well as the transcription factor Elk-1 are constitutively activated in esophagogastric RBM cell lines. To our surprise, the observed
activation of ERKs was not accompanied by MEK activation. Until now, the activated status of ERK has been shown to be predominantly accompanied by MEK activation (33, 34). One previous report has documented the importance in Swiss 3T3 fibroblasts of MEK activation for the initiation of MAPK activation by PDGF but also emphasize that prolonged MAPK activation by PDGF is MEK-independent (46). Other reports documenting constitutive ERK activation have shown it to be
micrometastatic RBM cell lines. PKC down-regulation was achieved by treating the cells with PMA (100 nM) for 48 h. The results shown are mean ± S.E. of six independent experiments. Significant (*) p < 0.05 changes from untreated cells are shown in C.

Although the specific role of ERKs in esophageal RBM cells is not known, it is reasonable to speculate that chronic activation of the ERK signaling cascade may influence the local metastatic environment to favor progression of esophageal metastatic cancer. Indeed, constitutive ERK activation has previously been shown to confer a tumorigenic and metastatic potential (48) as well as regulate cell motility and matrix metalloproteinase expression (49). Our data demonstrate that serine-threonine kinase Pak (p65Pak) a known effector of PI3K and PKC, however, both proved efficacious in significantly reducing esophageal RBM cell growth and suppressed the growth of colon tumors in vivo (51). Pharmacological inhibition of PI3K and PKC, however, both proved efficacious in significantly reducing esophageal RBM cell growth as well as ablating ERK activation (52). Our studies revealed that while PI3K seems to be necessary for ERK activation and esophageal metastatic cell growth, it does not require Raf activation as Raf was found not to be activated in these cell lines. Furthermore, upon examination of the K-ras gene in RBM cells, we did not detect the existence of point mutations. This is in agreement with a previous report demonstrating the absence of K-ras gene mutations in a series of cell lines established from esophageal cancer (2).

The involvement of PI3K lead us to investigate the role of the serine-threonine kinase Pak (p65Pak) a known effector of PI3K (25). The kinase-deficient Paks (Pak1R299 and Pak1L83,L86) were overexpressed in esophageal squamous cell carcinomas relative to normal tissue (47).

Abundant data indicate that constitutive activation of the ERK cascade increases the expression of PAC1, a member of the MAPK phosphatase family (14, 42) providing a pivotal role for this phosphatase in feedback inactivation of the stimulated ERK signaling pathway. PAC1 exhibits stringent substrate specificity for ERK and constitutive PAC1 expression inactivates ERK. While PAC1 expression is associated predominantly with hematopoietic cells (14), we have now shown that it is also present in CACO-2, HT-29, SW480, OC1, and -3 cells and can be up-regulated in the presence of serum. We failed, however, to detect PAC1 gene expression in RBM cell lines, thus ruling out the possibility of a role for this phosphatase in the observed ERK constitutive activation. Two other protein phosphatases, MKP-1 and MKP-2 (17, 18), may further explain the constitutive activation of ERKs in esophageal RBM cells. This activation by Pak was found to be sensitive to PI3K inhibition but not MEK inhibition. Although Pak is not usually associated with ERK activation, a recent report has shown that Pak can cooperate with Raf to activate ERK in a cross-cascade activation (26). It remains to be investigated whether Pak is interacting with Raf within RBM(1–5). However, the absence of MEK involvement in Pak1 signaling in RBM(1–5) ERK activation reveals a novel signaling pathway in these cells. Although Pak-induced JNK activation has also been previously demonstrated (26), our study shows neither JNK nor p38 were involved in the signaling cascade in RBM(1–5).

Both significantly reduced the phosphorylation levels of ERK1 and -2. Interestingly, we observed that hyperactive Pak (Pak1R299,L83,L86) could further enhance the activation state of ERKs in esophageal RBM cells. This activation by Pak was found to be sensitive to PI3K inhibition but not MEK inhibition. Although Pak is not usually associated with ERK activation, a recent report has shown that Pak can cooperate with Raf to activate ERK in a cross-cascade activation (26).

Although the specific role of ERKs in esophageal RBM cells is not known, it is reasonable to speculate that chronic activation of the ERK signaling cascade may influence the local metastatic environment to favor progression of esophageal metastatic cancer. Indeed, constitutive ERK activation has previously been shown to confer a tumorigenic and metastatic potential (48) as well as regulate cell motility and matrix metalloproteinase expression (49). Our data demonstrate that selective inhibition of the upstream kinase MEK does not affect esophageal RBM cell growth. This is in contrast to recent studies whereby inhibition of MEK attenuated the in vivo invasiveness of head and neck squamous cell carcinoma (50) and suppressed the growth of colon tumors in vivo (51). Pharmacological inhibition of PI3K and PKC, however, both proved efficacious in significantly reducing esophageal RBM cell growth as well as ablating ERK activation (52). Our studies revealed that while PI3K seems to be necessary for ERK activation and esophageal metastatic cell growth, it does not require Raf activation as Raf was found not to be activated in these cell lines. Furthermore, upon examination of the K-ras gene in RBM cells, we did not detect the existence of point mutations. This is in agreement with a previous report demonstrating the absence of K-ras gene mutations in a series of cell lines established from esophageal cancer (2).

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tion and its mechanistic basis require additional investigation, we may speculate that an abnormal down-regulation mechanism of ERKs may exist in RBM(1–5) involving MKP-1. In conclusion, although the complexity of the signaling cascade which converges on ERK seems daunting, delineation of this pathway may uncover new cellular targets for the pharmacological manipulation of esophageagastic metastatic RBM cells.

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Constitutive ERK1/2 Activation in Esophagogastric Rib Bone Marrow Micrometastatic Cells Is MEK-independent
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