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TITLE PAGE

Bradykinin B_2 Receptor Interacts with Integrin α5β1 to Transactivate Epidermal Growth Factor Receptor in Kidney Cells.

Inga I. Kramarenko, Marlene A. Bunni, John R. Raymond and Maria N. Garnovskaya

The Medical and Research Services of the Ralph H. Johnson Veterans Affairs Medical Center, and Department of Medicine (Nephrology Division) of the Medical University of South Carolina, Charleston, SC, 29425

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RUNNING TITLE PAGE

a) Running Title: *Integrin-GPCR Interaction in EGFR Transactivation*

b) Corresponding author: Dr. Maria N. Garnovskaya

Medical University of South Carolina,

Department of Medicine, Division of Nephrology,

96 Jonathan Lucas St., MSC 629, Charleston, SC, 29425-6290

Phone: 843-789-6774 or 843-876-5128;

Fax: 843-876-5129 or 843-792-8399

e-mail garnovsk@musc.edu

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Abstract – 247 words

Introduction – 743 words

Discussion – 1,335 words

d) ABBREVIATIONS: **GPCR**, G protein-coupled receptor; **BK**, bradykinin; **ERK**, extracellular signal-regulated protein kinase; **EGF**, epidermal growth factor; **EGFR**, epidermal growth factor receptor; **FAK**, focal adhesion kinase -non-receptor tyrosine kinase; **MAPK**, mitogen -activated protein kinase; **MEK**,

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mitogen and extracellular signal-regulated kinases kinase; **MMP**, matrix metalloproteinase; **mIMCD**, murine renal inner medullary collecting duct; **RGD,** (arginine-glycine-aspartic acid) sequence **PYK2**, a non-receptor tyrosine kinase.

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Abstract

We have shown previously that the vasoactive peptide bradykinin (BK) stimulates proliferation of a cultured murine cell model of the inner medullary collecting duct (mIMCD-3 cells) *via* transactivation of epidermal growth factor receptor (EGFR) by a mechanism that involves matrix metalloproteinases (collagenases-2 and -3). Because collagenases lack an integral membrane domain, we hypothesized that receptors for extracellular matrix proteins, integrins, may play a role in BK-induced signaling by targeting collagenases to the membrane, thus forming a functional signaling complex. BK-induced phosphorylation of extracellular signal-regulated protein kinase (ERK) in mIMCD-3 cells was reduced by ~65% by synthetic peptides containing an RGD (arginine-glycine-aspartic acid) sequence, supporting roles for integrins in BK-induced signaling. Neutralizing antibody against $\alpha5\beta1$ integrin partially (~60%) blocked BK-induced ERK activation but did not affect EGF-induced ERK activation. Silencing of $\alpha5$ and $\beta1$ expression by transfecting cells with small interfering RNAs (siRNA) significantly decreased BK-induced ERK activation (~80%) and EGFR phosphorylation (~50%). This effect was even more pronounced in cells that were co-transfected with siRNAs directed against both collagenases and $\alpha5\beta1$ integrin. Based on our results, we suggested that integrin $\alpha5\beta1$ is involved in BK-induced signaling in mIMCD-3 cells. Using immunoprecipitation/Western blotting, we demonstrated association of BK B_2 receptor with $\alpha5\beta1$ integrin upon BK treatment. Furthermore, BK induced association of $\alpha5\beta1$ integrin with EGFR. These data provide the first evidence that specific integrins are involved in BK B_2 receptor-induced signaling in kidney cells, and ultimately might lead to development of new strategies for treatment of renal tubulointerstitial fibrosis.

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Introduction

The vasoactive nonapeptide bradykinin (BK) plays important roles in the regulation of kidney functions such as electrolyte and water excretion (Mukai et al., 1996). Specifically, a role of BK in the control of absorptive function in the kidney collecting ducts is well established (Tomita et al., 1985; Zeidel et al., 1990). BK also acts directly as a potent cellular growth factor for multiple cell types including kidney cells. Previously, we established that the BK B_2 receptor stimulates early mitogenic signals associated with activation of extracellular signal-regulated protein kinase (ERK) in a murine epithelial cells derived from the inner medullary collecting duct (mIMCD-3 cells), and demonstrated that BK-induced cell proliferation depends on transactivation of the epidermal growth factor receptor (EGFR) (Mukhin et al., 2003). Further, we demonstrated that BK B_2 receptor-induced EGFR transactivation involves activation of matrix metalloproteinases (MMPs), namely collagenases-2 and -3 (Mukhin et al., 2006). Because collagenases lack an integral membrane domain, we hypothesized that integrins may play a role in BK-induced signaling by targeting collagenases to the membrane, thus forming a functional signaling complex. Integrins are heterodimeric receptors for cell-surface adhesion molecules and extracellular matrix (ECM) proteins, that are composed of two subunits, α and β. Each αβ combination has specific signaling properties (reviewed in Juliano, 2002). To date, eighteen α and eight β subunits have been identified, which form at least 24 different αβ integrins (Humphries et al, 2006).

The first interaction between integrins and MMPs was identified in melanoma cells in which it was demonstrated that the C-terminal domain of gelatinase-A (MMP-2)

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binds directly to integrin αVβ3, which localizes MMP-2 in a proteolytically active form on the surface of invasive cells (Brooks et al., 1996). Further, the involvement of the αVβ3/MMP-2 complex in tumor growth and angiogenesis has been demonstrated *in vivo* (Brooks et al., 1998). Integrin αVβ3 also cooperates with gelatinase-B (MMP-9) to regulate migration of breast cancer cells (Rolli et al., 2003). Purified β2 integrin has been shown to bind to the catalytic domain in pro-MMP-9 gelatinase to form complexes of pro-MMP-9 with both the αMβ2 and αLβ2 integrins in leukemic cell lines; these associations probably control the activation of the proenzyme (Stefanidakis et al., 2003). Cell surface interactions between β2 integrins and gelatinases plays roles in normal leukocyte migration and in cancer progression (Stefanidakis and Koivunen, 2006). Interactions with integrins also have been demonstrated for collagenase-1 (MMP-1). Thus, pro-MMP-1 specifically binds to α2β1 integrin on keratinocytes, facilitating the cleavage of type I collagen and keratinocyte migration (Dumin et al., 2001). This binding occurs via the I-domain of the α2 integrin subunit, and requires both the linker domain and the hemopexin-like domain of MMP-1 (Stricker et al., 2001). MMP-1 also interacts with α2β1 integrin in human neurons (Conant et al., 2004), and with α1β1 integrin in monocytes (Stricker et al., 2001). However, no interactions between integrins and collagenases 2 or 3 have been described.

Interesting connections between integrins and G protein-coupled receptors (GPCRs) have been described recently. Integrin-mediated cell anchorage impacts on GPCR signaling to the ERK/MAPK (Della Rocca et al., 1999; Short et al., 2000). Muscarinic receptors stimulate tyrosine phosphorylation of focal adhesion kinase (FAK) *via* an integrin-dependent mechanism (Slack, 1998). FAK phosphorylation in response

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to bombesin and muscarinic signaling depends on the integrity of the cytoskeleton (Hunger-Glaser et al., 2003). Activation of histamine receptors results in translocation of the non-receptor tyrosine kinase PYK2 to focal adhesions and enhances PYK2 tyrosine phosphorylation leading to ERK activation in HeLa cells (Litvak et al., 2000).

The precise molecular mechanism underlying integrin-mediated GPCR signaling to ERK remains to be defined, but one possibility involves integrin-mediated recruitment of cytoskeletal components to form a scaffold that facilitates efficient assembly of the components of the signaling pathway. Alternatively, some GPCRs interact directly with integrins: $P2Y_2$ nucleotide receptor contains an RGD motif in the first extracellular loop that enables it to interact with αVβ3 and αVβ5 integrins leading to increased astrocytes migration (Bagchi et al., 2005; Wang et al., 2005). In addition, β1 and β3 integrins co-localize with the mu opioid receptor in sensory neurons, and regulate receptor signaling probably by altering its coupling to either Gαi or Gαs proteins (Berg et al., 2007).

Possible interactions between the BK B_2 receptor and integrins have not been studied. Herein, we provide multiple lines of evidence that suggest the involvement of integrin α5β1 in BK-induced signaling, and describe physical and functional connections among BK B_2 receptor, α5β1 integrin and collagenases-2 and -3 in mIMCD-3 cells.

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Materials and Methods

Materials. Cell culture supplies were obtained from GIBCO/Invitrogen (Grand Island, NY), or Corning Costar (Cambridge, MA). Bradykinin, EGF and other reagent grade chemicals were from Sigma-Aldrich (St. Louis, MO). Cyclic RGD and control RGD peptides, and MMP inhibitors were from Calbiochem (La Jolla, CA). Phospho-ERK kit was obtained from Cell Signaling Technology (Beverly, MA). EGFR polyclonal antibody, anti-phospho-EGFR (Tyr^{1173}) monoclonal antibody, and monoclonal anti-phospho-tyrosine antibody were from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-BK B_2 receptor antibody was from BD Transduction Laboratories™ (Franklin Lakes, NJ). MMP-8 monoclonal antibody, MMP-13 monoclonal antibody, anti-α5β1 integrin polyclonal antibody, monoclonal blocking antibodies against α2β1, α5β1, αVβ6, and α6 integrins, antibodies against α2, α3, αV, and β1 integrin subunits and mouse monoclonal GAPDH antibody were from Chemicon International (Temecula, CA). Antibodies against integrin α2, α5, α6, β6 and β1 subunits, blocking antibodies against α3, αV and β3 integrins, MMP-8 siRNA, MMP-13 siRNA, integrin α2 siRNA, integrin α5 siRNA, integrin β1 siRNA and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). The RNA Stat-60 reagent was from Tel-Test, Inc., (Friendswood, TX). The RT^2 Profiler™ PCR Array System for Mouse Extracellular Matrix and Adhesion Molecules and the Oligo GEArray® kit for Mouse Extracellular Matrix and Adhesion Molecules were from SuperArray Bioscience Corporation (Frederick, MD).

Cell Culture. mIMCD-3 cells were obtained from American Type Culture Collection (Rockville, MD). mIMCD-3 cells were grown in equal mixtures of DMEM and Ham's

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F12, supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Study of expression levels of integrin subunits in mIMCD-3 cells. To monitor the expression profile of integrins in mIMCD-3 cells we employed the Oligo GEArray® kit for Mouse Extracellular Matrix and Adhesion Molecules. We extracted total RNA from mIMCD-3 cells that were grown on plastic plates, using the RNA Stat-60 reagent, and converted RNA into biotin-labeled cRNA target probes for Microarray hybridization using the TrueLabeling-AMP linear RNA amplification kit (SuperArray Bioscience Co). The cRNA targets (6 μg of cRNA) next were hybridized with oligonucleotide probes representing different genes that are printed on a nylon membrane. The resulting products on arrayed membranes were detected by a chemiluminescent detection kit, and analyzed by GEArray Analyzer data analysis software. We also used an RT^2 Profiler™ PCR Array System for mouse extracellular matrix and adhesion molecules that validates the expression of 84 relevant genes for cell-cell and cell-matrix interactions, including 9 α and 4 β integrin subunits. Total RNA from mIMCD-3 cells was extracted as above, and was converted to cDNA using the RT^2 Profiler™ PCR Array first strand kit. Quantitative real-time PCR was performed according to the manufacture's protocol. The presence of various integrin subunits in lysates from mIMCD-3 cells was supported by Western blots using commercially available antibodies.

Transfections of mIMCD-3 cells – Integrin and MMP silencing. Transfections of mIMCD-3 cells were achieved by nucleofection with an Amaxa Biosystems instrument (Giessen, Germany). 1×10^6 cells were resuspended in 100 μl of Nucleofector™

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solution R and nucleofected with either 100 nM of siRNA for MMP-8, MMP-13, or integrin α5β1 siRNA, or with the same amount of control siRNA using manufacturer's protocol T-16. 48 h post-nucleofection, cells were stimulated with BK, EGF or vehicle, lysed and analyzed for integrin and MMP expression by Western blotting with anti-MMP-8 or anti-MMP-13 or with anti-integrin α5, and β1 rabbit polyclonal antibodies, and for EGFR phosphorylation and ERK activation. Blots were re-probed with a mouse monoclonal GAPDH antibody to control for protein loading and for silencing specificity and efficiency.

ERK Assay. ERK phosphorylation was assessed by Western blot using a phosphorylation-state specific antibody that specifically recognizes threonine^{202} and tyrosine^{204}-phosphorylated ERK-1 and ERK-2 as previously described (Mukhin et al., 2003) in mIMCD-3 cells treated for 5 min with BK, EGF, or vehicle. The membranes were stripped using Re-Blot Plus antibody stripping solution (Chemicon International, Inc., Temecula, CA) and re-probed with the control ERK antibody, which recognizes equally well phosphorylated and non-phosphorylated ERK. Results are presented as intensities of phospho-ERK bands relative to total ERK bands and expressed as fold of basal phosphorylation (non-treated cells).

EGF Receptor Phosphorylation Assay. The phosphorylation state of EGFR was assessed by immunoprecipitation/Western blotting studies as previously described (Mukhin et al., 2003). Quiescent mIMCD-3 cells, grown in 100 mm dishes, were pretreated with vehicle or inhibitors for 30 min. Subseqently, cells were treated with 100 nM BK, 1 ng/ml EGF or vehicle for 5 minutes and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA,

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1 mM sodium fluoride, 1 mM Na_3VO_4, 1 μg/ml aprotinin, leupeptin, and pepstatin, each). Cell lysates were precleared by incubating with protein A-agarose bead slurries for 30 min at 4°C. Precleared lysates (1 μg/μl total cell protein) were incubated with 4 μg of anti-EGFR polyclonal IgG overnight at 4°C. The immunocomplexes were captured by the addition of protein A-agarose bead slurries, with incubation for two more hours at 4°C. The agarose beads were collected by centrifugation, washed three times with RIPA buffer, resuspended in 2× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. After wet transfer to polyvinylidine difluoride membranes, the membranes were probed with monoclonal anti-phospho-EGFR (Tyr^{1173}) antibody to assess the phosphorylation state of EGFR, or with EGFR antibody.

Studies of possible complex formation between integrin α5β1, BK B_2 receptor and collagenases. Quiescent mIMCD-3 cells, grown in 100 mm dishes, were treated with 100 nM of BK, 1 ng/ml EGF or with vehicle for different time periods, and lysed in RIPA buffer. Cell lysates were pre-cleared by incubating with protein A-agarose bead slurries for 30 min at 4°C. Pre-cleared lysates (1 μg/μl total cell protein) were incubated with 4 μg of anti-α5β1 integrin polyclonal IgG overnight at 4°C. The immunocomplexes were captured by addition of protein A-agarose bead slurry and incubation for 2 more hours at 4°C. The agarose beads were collected by centrifugation, washed 3 times with RIPA buffer, resuspended in 2× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. After wet transfer to polyvinylidine difluoride membranes, the membranes were probed with monoclonal anti-BK B_2 receptor, with monoclonal anti-MMP-8 or anti-MMP-13 antibody, and with anti-EGFR antibody to study possible physical associations of these proteins.

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Data Analysis. ERK assays were performed in duplicate and repeated at least 3 separate times. EGFR assays were repeated 3 separate times for each condition. Data are presented as mean + S.E.M. (Standard Error of the Mean), and were analyzed for repeated measures by Student's t-test for unpaired 2-tailed analysis, and by an ANOVA test. Differences were considered significant at $p < 0.05$. Statistical probability (p) in figures expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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Results

Integrins are involved in BK-induced ERK1/2 activation and EGFR transactivation in mIMCD-3 cells.

In the first set of experiments we tested the involvement of integrins in B_2 receptor-induced EGFR transactivation and ERK phosphorylation using arginine-glycine-aspartic acid (RGD) peptides. RGD was originally identified as the sequence in fibronectin that is a recognition site for α5β1 integrin, but it also serves as a recognition motif for other integrins including α3β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6 and α2bβ3 (Plow et al, 2000). Quiescent mIMCD-3 cells were pretreated for one hour with 28 μM of either control or cyclic RGD peptides prior to stimulation with 100 nM BK or with 1 ng/ml EGF for 5 minutes. Control RGD peptides were without effect whereas cyclic RGD peptides reduced the activation of ERK by BK by ~65% suggesting that integrins with RGD recognition specificity may be involved in BK-induced ERK phosphorylation (**Figure 1A**). At the same time, cyclic RGD peptides did not affect EGF-induced ERK activation (**Figure 1C**).

Expression profile of integrin subunits in mIMCD-3 cells.

Having established a probable role for integrins in BK-induced signaling in mIMCD-3 cells, we attempted to examine specific integrins that could mediate this response. In order to plan more specific experiments, we needed to determine which integrins are present in mIMCD-3 cells because at least eighteen α and eight β integrin subunits have been identified. First, we employed the Oligo GEArray® kit for Mouse Extracellular Matrix and Adhesion Molecules, which represents 113 genes encoding proteins important for the attachment of cells to their surroundings including various

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types of cell adhesion molecules (such as the integrins, IgG superfamily members, cadherins and catenins, and selectins) as well as extracellular matrix proteins, proteases (such as the matrix metalloproteinases and the serine and cysteine proteinases) and their inhibitors. This array allowed us to determine simultaneously the expression profile of 13 α and 7 β integrin subunits in mIMCD-3 cells. **Table 1** summarizes mRNA expression of integrins in mIMCD-3. Integrin subunits α2b, α3, αV and β1 appeared to be the most abundant in mIMCD-3 cells. The messages for α2, α6, α7, αX, β6 and β7 integrin subunits also were detectable. To our surprise we did not detect message for α5 integrin subunit although we expected to find α5β1 integrin in mIMCD-3 cells based on the ability of RGD peptides to affect BK-induced signaling (**Figure 1A**).

To verify the microarray data, we employed an RT^2 Profiler™ PCR Array System for mouse extracellular matrix and adhesion molecules that measurees the expression of 84 relevant genes for cell-cell and cell-matrix interactions including nine α (α2, α3, α4, α5, αE, αL, αM, αV and αX) and four β (β1, β2, β3 and β4) integrin subunits. The expression of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as control. RT-PCR results confirmed the expression of α2, α3, αX, αV and β1 genes identified by the Oligo microarray (not shown). In addition, an RT^2 Profiler™ PCR Array System allowed us to detect the message for α5 integrin subunit. Our inability to detect the message for α5 integrin subunit by the Oligo GEArray could be caused by a technical issue with this particular Oligo GEArray layout, because the message for α5 integrin (spot #48) is probably masked by a strong message for Fibronectin-1 (spot #40) which is highly expressed in mIMCD-3 cells. Further, to confirm the mRNA data and to

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support the expression of integrins on a protein level we performed Western blotting on mIMCD-3 lysates using commercially available antibodies against integrins. Results presented in **Figure 2** support the presence of integrins α2, α3, α5, α6, αV, β1 and β6 in mIMCD-3 cells.

Roles of specific integrins in BK-induced ERK1/2 activation in mIMCD-3 cells.

To examine specific integrins that are involved in BK-induced ERK phosphorylation, we employed commercially available neutralizing antibodies against mouse integrin subunits. Quiescent mIMCD-3 cells were pretreated for 2 hours with anti-integrin antibodies or normal rabbit IgG prior to stimulation with 100 nM BK or with 1 ng/ml EGF for 5 minutes. Neutralizing antibody against α5β1 integrin when used in a concentration of 100 ng/ml significantly (~60%) blocked BK-induced phosphorylation of ERK in mIMCD-3 cells (**Figure 1B**; n = 3, $p < 0.001$), but did not affect EGF-induced ERK activation (**Figure 1D**). Neutralizing antibody against α2β1 integrin in some experiments also partially (~25%) decreased BK-induced phosphorylation of ERK in mIMCD-3 cells, although these results were not statistically significant (not shown). At the same time, neutralizing antibodies against α3, αV, β6 and αVβ6 were without effect (not shown), suggesting specificity in the involvement of α5β1 integrin in BK-induced ERK phosphorylation.

Transfection of mIMCD-3 cells with integrin and MMP siRNAs decreases BK-induced ERK activation.

To further support the involvement of integrins in BK-induced ERK activation, we employed RNA-mediated interference to knock down the expression of α5β1 integrin. mIMCD-3 cells were nucleofected with either 100 nM of siRNA for integrin α5β1 or with

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the same amount of control siRNA. 48 hours postnucleofection, cells were stimulated with vehicle or 100 nM BK, or 1 ng/ml EGF for 5 minutes, lysed, and analyzed for ERK phosphorylation. Cells transfected with α5β1 integrin siRNAs demonstrated ~80% less BK-induced ERK activation than cells transfected with control siRNA (**Figure 3A**). EGF-induced ERK activation was not affected by α5β1 integrin down-regulation (**Figure 3B**). Silencing of α2β1 integrin did not affect either BK-induced or EGF-induced phosphorylation of ERK in mIMCD-3 cells (not shown), suggesting specificity in the involvement of α5β1 integrin in BK-induced ERK phosphorylation.

Because previously we demonstrated the involvement of MMP-8 and MMP-13 in BK-induced ERK activation (Mukhin et al, 2006), we performed the combined inhibition of MMP-8 or MMP-13 and α5β1 integrins by co-transfecting cells with MMP-8 or MMP-13 siRNA in addition to integrin siRNAs. Cells transfected with a combination of MMP-8 and α5β1 integrin siRNAs demonstrated ~75% decrease in BK-induced ERK phosphorylation (**Figure 3A**). Cells transfected with a combination of MMP-13 and α5β1 integrin siRNAs demonstrated ~90% decrease in BK-induced ERK phosphorylation. Because in our previous study we proposed roles for both collagenases in BK-induced ERK phosphorylation (Mukhin et al, 2006), we blocked both MMP-8 and MMP-13 by transfecting cells with a combination of MMP-8 and MMP-13 siRNAs in addition to silencing α5β1 integrin. Each set of transfected cells demonstrated significant decreases in BK-induced ERK phosphorylation compared with the set transfected with scrambled siRNA. Although differences between cells transfected with α5β1 integrin siRNA only and cells co-transfected with MMP-8 and MMP-13 siRNAs in addition to α5β1 integrin siRNA, were not statistically significant, in

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all experiments there was a trend that down-regulation of both collagenases in addition to α5β1 integrin resulted in a complete inhibition of the signal. Down-regulation of α5β1 integrin either alone or in combination with MMP-8 and/or MMP-13 did not affect EGF-induced ERK activation (**Figure 3B**). Effective silencing of integrin and MMP expression in mIMCD-3 cells transfected with siRNAs was supported by immunoblotting (**Figure 4C**). These results support the hypothesis that integrin α5β1 most likely mediates BK-induced signaling in mIMCD-3 cells.

Transfection of mIMCD-3 cells with integrin α5β1 and MMP-13 and MMP-8 siRNAs decreases BK-induced EGFR phosphorylation.

Because BK activates ERK in mIMCD-3 cells via EGFR transactivation (Mukhin et al., 2003), in the next series of experiments we assessed the involvement of α5β1 integrin in BK-induced EGFR phosphorylation. To test the involvement of integrin α5β1, we transfected mIMCD-3 cells with either 100 nM of integrin α5β1 siRNA or with control (scrambled) siRNA. To block the activity of collagenases we silenced MMP-8 and/or MMP-13 expression by nucleofection with MMP-8 siRNA, MMP-13 siRNA or with both MMP-8 and MMP-13 siRNAs. 48 h postnucleofection, cells were stimulated with vehicle or 100 nM BK or with 1 ng/ml EGF for 5 min, lysed, and analyzed for EGFR phosphorylation as described in Methods. Silencing of integrin α5β1 significantly ($p < 0.01$) decreased BK-induced EGFR phosphorylation (**Figure 4A**). At the same time, the combined inhibition of MMP-8 and α5β1 integrin significantly blocked BK-induced EGFR phosphorylation by ~70%, and the combined inhibition of MMP-13 and α5β1 integrin completely abolished the effect of BK (**Figure 4**). Similar results were obtained when we blocked the activity of collagenases by pretreatment mIMCD-3 cells transfected integrin

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α5β1 siRNA with chemical inhibitors of MMP-8 and MMP-13 (not shown) suggesting that both MMP-8, MMP-13, as well as α5β1 integrin are involved in BK-induced phosphorylation of EGFR. However, differences in BK-induced EGFR phosphorylation between cells transfected with α5β1 integrin siRNA only and cells co-transfected with MMP-8 and MMP-13 siRNAs in addition to α5β1 integrin siRNA were not statistically significant. At the same time, down-regulation of α5β1 integrin either alone or in combination with MMP-8 and/or MMP-13 did not affect EGF-induced EGFR phosphorylation (**Figure 4B**).

BK induces complex formation between α5β1 integrin, EGFR, MMP-13, MMP-8, and BK B_2 receptor.

We next considered that BK stimulates the assembly of a signal transduction complex which includes molecules involved in EGFR phosphorylation, and examined whether BK could induce a physical interaction between α5β1 integrin, EGFR, and collagenases. To explore this possibility, we used immunoprecipitation of lysates from cells treated with vehicle or BK or EGF with anti-α5β1 integrin antibody, followed by Western blotting with antibody against EGFR, MMP-8, MMP-13 and BK B_2 receptor. **Figure 5A** shows that α5β1 integrin and EGFR (175 kDa) co-immunoprecipitate and that their association can be increased by stimulation of mIMCD-3 cells with BK. Stimulation with EGF did not significantly increase association between α5β1 integrin and EGFR, supporting that α5β1 integrin is not essential for EGF-induced EGFR activation. **Figure 5B** shows that immunoprecipitation of α5β1 integrin from mIMCD-3 cells treated with BK but not with EGF, resulted in co-precipitation of the BK B_2 receptor (42 kDa). The BK B_2 receptor antibody, which we employed for these studies has been used previously used by our

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group (Kramarenko et al, 2009) and others (Golser et al, 2000; Xie et al, 2000) to demonstrate the presence of the endogenous and/or transfected BK B_2 receptor in different cell lines by Western blotting, and also to immunoprecipitate BK B_2 receptor (Golser et al, 2000). The bands corresponding to EGFR or BK B_2 receptor were not detectable in precipitates obtained from identical samples precipitated in the presence of normal rat immunoglobulins instead of α5β1 integrin antibody (data not shown). These results suggest that BK induces co-precipitation of α5β1 integrin with EGFR and BK B_2 receptor. Furthermore, we found that MMP-8 and MMP-13 also are present in BK-induced signaling complex (not shown).

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Discussion

The current work describes a novel mechanism of EGFR transactivation by the Gq-coupled BK B_2 receptor in mIMCD-3 cells that involves integrin $\alpha5\beta1$. What is new about this work is that we have (1) characterized the repertoire of integrins in mIMCD-3 cells using RT-PCR, microarray detection and Western blotting; (2) implicated integrins as key mediators of BK-induced ERK activation using RGD peptides; (3) provided evidence that integrin $\alpha5\beta1$ is involved in BK-induced EGFR transactivation based on results of experiments utilizing neutralizing anti-integrin antibodies and siRNA; and (4) demonstrated for the first time that BK induces formation of a signaling complex among $\alpha5\beta1$ integrin, EGFR, the BK B_2 receptor, and probably MMP-13, MMP-8.

In our previous work we established that the BK B_2 receptor stimulates early mitogenic signals associated with activation of ERK1/2 in mIMCD-3 cells, and demonstrated that BK-induced cell proliferation was dependent on activation of EGFR (Mukhin et al., 2003). Further, we described a novel mechanism of EGFR transactivation by the Gq-coupled BK B_2 receptor that involves activation of matrix metalloproteinases (MMPs), namely collagenases 2 and 3 (MMP-8 and MMP-13). We demonstrated that collagenases 2 and 3 are activated by the BK B_2 receptor in mIMCD-3 cells, and are involved in cross talk between the B_2 receptor and EGFR (Mukhin et al., 2006). In the current study, we looked further into this mechanism by testing the hypothesis that in mIMCD-3 cells, integrins may play a role in BK-induced signaling by targeting collagenases to the membrane, thus forming a functional signaling complex. Several GPCRs have been shown to activate ERK in an integrin-dependent manner: thrombin, BK and lysophosphatidic acid (LPA) receptors in PC12 rat

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pheochromocytoma cells (Della Rocca et al.,1999); gonadotropin-releasing hormone receptors (GnRHR) expressed in HEK293 cells (Davidson et al., 2004); P2Y receptors in endothelial cells (Short et al., 2000); histamine receptors in HeLa cells (Litvak et al., 2000); and δ- opioid receptors transfected into HEK293 cells and endogenously expressed in neuroblastoma x glioma hybrid NG108-15 cells (Eisinger and Ammer, 2008).

In our study we assessed the involvement of integrins in BK-induced signaling in mIMCD-3 cells, using RGD-containing synthetic peptides that inhibit ligand binding to integrins with RGD recognition specificity, e.g. integrins α3β1, α5β1, α8β1, αVβ1, αVβ3, αVβ5, αVβ6, and α2bβ3. RGD peptides decreased BK-induced ERK activation by ~65% (**Figure 1A**) without affecting EGF-induced ERK activation (**Figure 1C**) supporting the hypothesis that integrins are involved in BK-induced ERK phosphorylation in mIMCD-3 cells. Next we aimed to determine specific integrins responsible for mediating BK-induced ERK activation. Using two different methods of mRNA analysis and Western blotting, we established that mIMCD-3 cells express the following integrin subunits: α2, α2b, α3, α5, αV, αX, β1 and β6 (**Table and Figure 2**). These subunits may form four integrins with RGD recognition specificity: α3β1, α5β1, αVβ1 and αVβ6. We employed neutralizing antibodies against these integrins to study their possible involvement in BK-induced ERK activation. Neutralizing antibody against α5β1 integrin significantly blocked BK-induced phosphorylation of ERK by ~60% in mIMCD-3 cells without affecting EGF-induced ERK activation (**Figure 1B,D**), suggesting the involvement of α5β1 intergin in BK-induced ERK phosphorylation. At the same time, neutralizing antibodies against α3, αV, β6 and αVβ6 integrins did not change BK-induced ERK

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phosphorylation suggesting lack of involvement of α3β1, αVβ1 and αVβ6 integrins. We further supported the involvement of α5β1 integrin in BK-induced signaling by using mIMCD-3 cells, in which the expression of α5 and β1 subunits was knocked down by an RNA-mediated interference (**Figure 3A**). Because silencing of integrin α5β1 also decreased BK-induced EGFR phosphorylation (**Figure 4A**), the present findings indicate that BK-induced ERK activation is mediated by integrin-stimulated EGFR. At the same time, down-regulation of α5β1 integrin either alone or in combination with MMP-8 and/or MMP-13 did not affect EGF-induced EGFR phosphorylation and ERK activation (**Figures 3B, 4B**).

Thus, for the first time we have identified a specific integrin (α5β1) that specifically mediates BK-induced EGFR transactivation and ERK phosphorylation in mIMCD-3 cells.

The ability of integrins to cooperate with receptor tyrosine kinases including EGFR to transduce proliferative signals and to regulate cell survival and migration has been discussed previously (Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002). Integrins are able to form physical complexes with EGFR at the cell membrane and to trigger ligand-independent phosphorylation of Tyr^{845}, Tyr^{1068}, Tyr^{1086}, and Tyr^{1173} residues in the EGFR molecule (Moro et al., 1998). This integrin-dependent EGFR activation appears necessary for full EGFR-dependent transcriptional responses (Cabodi et al., 2004). GPCR-dependent transactivation of EGFR is usually mediated by MMP-dependent processing of membrane EGFR pro-ligands (Prenzel et al., 1999). In our previous study we demonstrated the involvement of MMP-8 and MMP-13 in BK-induced EGFR transactivation in mIMCD-3 cells but at the same time we found that this

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transactivation does not require extracellular release of EGF-like growth factors such as HB-EGF and/or TGF-α (Mukhin et al., 2006). Our present data favor the possibility that BK induces association of EGFR with integrin α5β1, thus causing ligand-independent phosphorylation and activation of EGFR. In support of that idea, we showed that α5β1 integrin and EGFR co-immunoprecipitate upon BK treatment (**Figure 5A**).

The concept that the association of MMPs with integrins can modify intracellular signaling has been demonstrated in several cell models (reviewed in Stefanidakis and Koivunen, 2006). However most reports describe interactions between integrins and gelatinases (MMP-2 and MMP-9) either in tumor cells (Brooks et al., 1998; Rolli et al., 2003) or in leukocytes (Stefanidakis et al., 2003). The only collagenase described to interact with integrins, collagenase-1 (MMP-1), was reported to be associated with α2β1 integrin in keratinocytes (Dumin et al., 2001) and in human neurons (Conant et al., 2004), and with α1β1 integrin in monocytes (Stricker et al., 2001). In the current work the involvement of MMP-8 and MMP-13 in BK-induced ERK activation was supported by combined inhibition of MMP-8 and/or MMP-13 and α5β1 integrin by co-transfecting cells with MMP-8 or MMP-13 siRNA in addition to integrin siRNAs. Although differences between cells transfected with α5β1 integrin siRNA only and cells co-transfected with MMP-8 and MMP-13 siRNAs in addition to α5β1 integrin siRNA, were not statistically significant, in all experiments there was a trend that down-regulation of both collagenases in addition to α5β1 integrin resulted in a complete inhibition of BK-induced EGFR phosphorylation and ERK activation. (**Figures 3A and 4A**). Thus, the current work suggests that collagenases-2 and -3 (MMP-8 and MMP-13) may act in concert with integrin α5β1 to mediate BK-induced phosphorylation of EGFR and ERK in

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kidney cells. Interestingly, the BK B_2 receptor also was co-immunoprecipitated with α5β1 intergin after BK treatment of mIMCD-3 cells (**Figure 5B**).

This work is novel in that there are only a few reports regarding interactions between GPCRs and integrins. Colocalization of β1 and β3 integrins with the mu opioid receptor was detected in trigeminal ganglion neurons by immunocytochemistry and confocal imaging (Berg et al., 2007). These RGD-binding integrins probably regulate the spatial distribution of G proteins in plasma membrane microdomains containing GPCRs; therefore, the relative amounts of activated integrins (β1 or β3) at focal adhesions govern G protein subunit composition (Gαi versus Gαs) coupled to the mu opioid receptor, thus regulating the signaling of this receptor in sensory neurons. The only GPCR that has been shown to interact directly with integrins, the $P2Y_2$ nucleotide receptor, contains an RGD motif in the first extracellular loop that enables it to interact with αVβ3 and αVβ5 integrins (Erb et al., 2001). These interactions between the $P2Y_2$ nucleotide receptor and αV integrins are necessary for the receptor to activate Go and to initiate Go-mediated signaling events leading to chemotaxis (Bagchi et al., 2005), and also are critical for astrocyte migration (Wang et al., 2005). Our studies provide the first evidence of the interaction of the BK B_2 receptor with α5β1 integrin, and demonstrate that this interaction leads to transactivation of EGFR, and ERK phosphorylation in cultured mIMCD-3 cells.

In conclusion, these studies demonstrate a novel mechanism of EGFR transactivation by the Gq-coupled BK B_2 receptor that involves formation of a functional complex between α5β1 integrin, EGFR and BK B_2 receptor.

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References

Bagchi S, Liao Zh, Gonzales FA, Chorna NE, Seye CI, Weisman GA, Erb L. (2005) The $P2Y_2$ nucleotide receptor interacts with αV integrins to activate G_o and induce cell migration. *J Biol Chem* **280**: 39050-39057.

Berg KA, Zardeneta G, Hargreaves KM, Clarke WP, Milam SB. (2007) Integrins regulate opioid receptor signaling in trigeminal ganglion neurons. *Neuroscience* **144**: 889-897.

Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresh DA. (1996) Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* **85**: 683-693.

Brooks PC, Sillettu S, von Schalscha TL, Friedlander M, Cheresh DA. (1998) Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. *Cell* **92**: 391-400.

Cabodi S, Moro L, Bergatto E, Erba E, Di Stefano P, Turco E, Tarone G, Defilippi P. (2004) Integrin regulation of epidermal growth factor (EGF) receptor and of EGF-dependent responses. *Biochemical Soc Transactions* **32**: 438-442.

Conant K, Hillaire C St, Nagase H., Visse R, Gary D, Haughey N, Anderson C, Turchan J, Nath A. (2004) Matrix metalloproteinase 1 interacts with neuronal integrins and stimulates dephosphorylation of Akt. *J Biol Chem* **279**: 8056-8062.

Davidson L, Pawson AJ, Millar RP, Maudsley S. Cytoskeletal reorganization dependence of signaling by the gonadotropin-releasing hormone receptor. (2004) *J Biol Chem* **279**: 1980-1993.

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Molecular Pharmacology Fast Forward. Published on April 12, 2010 as DOI: 10.1124/mol.110.064840
This article has not been copyedited and formatted. The final version may differ from this version.

Della Rocca GJ, Maudsley S, Daaka Y, Lefkowitz RJ, Luttrell LM. (1999) Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. *J Biol Chem* **274**: 13978-13984.

Dumin JA, Dickeson SK, Stricker TP, Bhattacharyya-Pakrasi M, Roby JD, Santoro SA, Parks WC. (2001) Pro-collagenase-1 (Matrix Metalloproteinase-1) binds the α2β1 integrin upon release from keratinocytes migrating on type I collagen. *J Biol Chem* **276**: 29368-29374.

Eisinger DA, Ammer H. δ- Opioid receptors activate ERK/MAP kinase via integrin-stimulated receptor tyrosine kinases. (2008) *Cellular Signaling* **20**:2324-2331.

Erb L, Liu J, Ockerhausen J, Kong Q, Garrad RC, Griffin K, Neal C, Krugh B, Santiago-Pérez L, González F, Gresham H, Turner JT, and Weisman GA. (2001) An RGD sequence in the P2Y(2) receptor interacts with alpha(V)beta(3) integrins and is required for G (o)-mediated signal transduction. *J Cell Biol* **153**: 491–501.

Golser R, Gorren A, Leber A, Andrew P, Habisch H-J, Werner ER, Schmidt K, Venema RC, Mayer B. (2000) Interaction of endothelial and neuronal nitric-oxide synthases with the bradykinin B2 receptor. *J Biol Chem,* **275**, 5291-5296.

Humphries JD, Byron A, and Humphries MJ. (2006) Integrin ligands at a glance. *J Cell Sci* **119**:3901-3903.

Hunger-Glaser I, Salazar EP, Sinnett-Smith J, Rozengurt E. (2003) Bombesin, lysophosphatidic acid, and epidermal growth factor rapidly stimulate focal adhesion kinase phosphorylation at Ser-910: requirement for ERK activation. *J Biol Chem* **278**:22631-22643.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 19, 2024

Molecular Pharmacology Fast Forward. Published on April 12, 2010 as DOI: 10.1124/mol.110.064840
This article has not been copyedited and formatted. The final version may differ from this version.

Juliano RL. (2002) Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. *Annu Rev Pharmacol Toxicol* **42**:283-323.

Kramarenko I, Bunni MA, Morinelli TA, Raymond JR, Garnovskaya MN. (2009) Identification of a functional bradykinin B_2 receptors endogenously expressed in HEK293 cells. *Biochem Pharmacol* **77**; 269-276.

Litvak V, Tian D, Shaul YD, and Lev S. (2000) Targeting of PYK2 to focal adhesions as a cellular mechanism for convergence between integrins and G protein-coupled receptor signaling cascades. *J Biol Chem* **275**: 32736-32746.

Miranti CK, Brugge JS. (2002) Sensing the environment: A historical perspective on integrin signal transduction. *Nature Cell Biology* **4**: E83-E90.

Moro L, Venturino M, Bozzo C, Silengo L, Altruda F, Beguinot L, Tarone G, Defilippi P. (1998) Integrins induce activation of EGF receptor: Role in MAP kinase induction and adhesion-dependent cell survival *EMBO J.* **17**: 6622-6632.

Mukai H, Fitzgibbon WR, Bozeman G, Margolius HS, Ploth DW. (1996) Bradykinin B_2 receptor antagonist increases chloride and water absorption in rat medullary collecting duct. *Am J Physiol* **271**: R352-R360.

Mukhin YV, Garnovsky, E.A., Ullian, M.E., and Garnovskaya MN. (2003) Bradykinin B_2 receptor activates extra-cellular signal regulated protein kinase in mIMCD-3 cells via epidermal growth factor receptor transactivation. *J Pharmacol Exp Ther* **304**: 968-977.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 19, 2024

Molecular Pharmacology Fast Forward. Published on April 12, 2010 as DOI: 10.1124/mol.110.064840
This article has not been copyedited and formatted. The final version may differ from this version.

Mukhin YV, Gooz M, Raymond JR, and Garnovskaya MN. (2006) Collageneses 2 and 3 mediate epidermal growth factor receptor transactivation by Bradykinin B_2 receptor in kidney cells. *J Pharmacol Exp Ther* **318**: 1033-1043.

Plow ED, Haas TA, Zhang L, Loftus J, and Smith JW. (2000) Ligand binding to integrins. *J Biol Chem* **275**: 21785-21788.

Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A. (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**: 884-888.

Rolli M, Fransvea E, Pilch J, Saven A, Felding-Habermann B. (2003) Activated integrin αvβ3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells. *Proc Natl Acad Sci USA* **100**: 9482-9487.

Schwartz MA, Ginsberg MH. (2002)Networks and crosstalk: Integrin signalling spreads. *Nature Cell Biology* 2002 **4**:E65-E68.

Short SM, Boyer JL, Juliano RL. (2000) Integrins regulate the linkage between upstream and downstream events in G protein-coupled receptor signaling to mitogen-activated protein kinase. *J Biol Chem* **275**: 12970-12977.

Slack BE. (1998) Tyrosine phosphorylation of paxillin and focal adhesion kinase by activation of muscarinic m3 receptors is dependent on integrin engagement by the extracellular matrix. *Proc Natl Acad Sci USA* **95**: 7281-7286.

Stefanidakis M, Björklund M, Ihanus E, Gahmberg CG, and Koivunen E. (2003) Identification of a negatively charged peptide motif within the catalytic domain of progelatinases that mediates binding to leukocyte β2 integrins *J Biol Chem* **278**: 34674 – 34684.

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Molecular Pharmacology Fast Forward. Published on April 12, 2010 as DOI: 10.1124/mol.110.064840
This article has not been copyedited and formatted. The final version may differ from this version.

Stefanidakis M and Koivunen E. (2006) Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression. *Blood* **108**: 1441 - 1450.

Stricker TP, Dumin JA, Dickeson SK, Chung L, Nagase H, Parks WC, Santoro SA. (2001) Structural analysis of the α2 integrin I domain/procollagenase-1(Matrix Metalloproteinase-1) interaction. *J Biol Chem* **276**: 29375-29381.

Tomita K, Pisano JJ, Knepper MA. (1985) Control of sodium and potassium transport in the cortical collecting duct of the rat. Effects of bradykinin, vasopressin, and deoxycorticosterone. *J Clin Invest* **76**: 132-136.

Wang M, Kong Q, Gonzalez FA, Sun G, Erb L, Seye C, Weisman GA. (2005) P2Y2 nucleotide receptor interaction with alphaV integrin mediates astrocyte migration. *J Neurochem* **95**: 630-640.

Xie P, Browning DD, Hay N, Mackman N, Ye RD. (2000) Activation of NF-κB by Bradykinin through a $G\alpha_q$- and Gβγ-dependent pathway that involves Phosphoinositide 3-Kinase and Akt. *J Biol Chem*, **275**, 24907-24914.

Zeidel ML, Jabs K, Kikeri D, Silva P. (1990) Kinins inhibit conductive Na^+ uptake by rabbit inner medullary collecting duct cells. *Am J Physiol* **258**: F1584-F1591.

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Footnotes

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b) The preliminary results of this work were presented previously at the 2008 Experimental Biology Meeting, San Diego, CA. [Kramarenko I, Bunni M, Raymond JR, and Garnovskaya MN. (2008) Bradykinin B_2 receptor interacts with integrin α5/β1 to transactivate epidermal growth factor receptor in kidney cells. *The FASEB Journal.* **22**:829.5].

c) Address correspondence to: Dr. Maria N. Garnovskaya,

Department of Medicine – Nephrology, Medical University of South Carolina,

96 Jonathan Lucas St., MSC 629, Charleston, SC, 29425-6290

Phone: 843-789-6774 or 843-876-5128

Fax: 843-876-5129 or 843-792-8399

E-mail garnovsk@musc.edu

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Figure 1: Involvement of integrins in BK-induced ERK phosphorylation

Quiescent mIMCD-3 cells were pretreated for one hour either with 28 μM control or cyclic RGD peptides prior to stimulation with 100 nM BK (**A**) or with 1 ng/ml EGF (**C**) for 5 minutes.

Quiescent mIMCD-3 cells were pretreated for 2 hours with 0.1 mg/ml neutralizing anti-integrin antibodies prior to stimulation with 100 nM BK (**B**) or with 1 ng/ml EGF (**D**) for 5 minutes. Control samples were preincubated with 0.1 mg/ml of normal rabbit IgG.

ERK phosphorylation was measured as described in Methods. Bars represent intensities of phospho-ERK bands relative to total ERK expressed as fold of basal (cells treated with vehicle). Experiments were performed 3 times in duplicate. Data are presented as mean + S.E.M. Statistical probability (p) expressed as $^{***}p < 0.001$.

Table: mIMCD-3 cells express mRNA for α- and β-integrins

Expression of mRNAs for specific integrins was assessed by by Oligo GEArray® as described in Materials and Methods.

Figure 2: Western blot analysis of mIMCD-3 lysates

Western blot analyses of mIMCD-3 lysates (40 μg of total protein) were performed with commercially available anti-integrin antibodies to demonstrate the expression of these integrins on a protein level. The bands of α2 (155 kDa), α3 (150 and 130 kDa), α5 (150 kDa), α6 (140 kDa), αV (150 kDa), β1(130 kDa), and β6 (97 kDa) are indicated.

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Figure 3: Transfection of mIMCD-3 cells with integrin and MMP siRNAs decreases BK-induced ERK activation

mIMCD-3 cells were nucleofected either with 100 nM of siRNA for integrin α5β1 alone (-α5β1), or with combinations of MMP-8 siRNA (-α5β1 – MMP-8) and/or MMP-13 siRNA (-α5β1 – MMP-13), or with combinations of all siRNAs (-α5β1- MMP-8-MMP-13), or with the same amount of control siRNA (control). 48 hours post-nucleofection, cells were stimulated with vehicle or 100 nM BK (**A**) or with 1 ng/ml EGF (**B**) for 5 minutes, lysed, and analyzed for ERK phosphorylation.

ERK phosphorylation was measured as described in Methods. Bars represent intensities of phospho-ERK bands relative to total ERK expressed as fold of basal (cells treated with vehicle). Experiments were performed 3 times in duplicate. Data are presented as mean + S.E.M. Statistical probability (*p*) expressed as $^{**}p < 0.01$ compared with control BK-treated cells. ANOVA (-α5β1) compared with (-α5β1 – MMP-8), (-α5β1 – MMP-13) or with (-α5β1- MMP-8-MMP-13), not significant.

Figure 4: Transfection of mIMCD-3 cells with integrin α5β1 and MMPs siRNAs decreases BK-induced EGFR phosphorylation

Cells were nucleofected with either 100 nM of α5β1 siRNA (-α5β1), or with a combination of MMP-8 siRNA (-α5β1 – MMP-8), or MMP-13 siRNA (-α5β1 – MMP-13), or with a combination of all siRNAs (-α5β1- MMP-8-MMP-13), or with the same amount of control siRNA (control), as described in Methods. 48 hours post-nucleofection, cells were stimulated with vehicle or 100 nM BK (**A**) or with 1 ng/ml EGF (**B**) for 5 minutes, lysed, and analyzed for EGFR phosphorylation as described in Methods. Experiments were performed at least 3 times. Data are presented as mean + S.E.M. $^{**}p < 0.01$

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compared with control BK-treated cells. ANOVA (-α5β1) compared with (-α5β1 – MMP-8), (-α5β1 – MMP-13) or with (-α5β1- MMP-8-MMP-13), not significant.

C. Western blot analyses of lysates of mIMCD-3 cells transfected with either scrambled siRNA or siRNAs for α5, β1, MMP-8 and MMP-13 (40 µg of total protein) were performed with commercially available antibodies against α5 and β1 integrin subunits, MMP-8 and MMP-13 to demonstrate down-regulation of these proteins. Blots were stripped and re-probed with antibody against GAPDH to control for the specificity of silencing and protein loading.

Figure 5: BK induces complex formation between EGFR and α5β1 integrin

Lysates from mIMCD-3 cells treated with vehicle, or 100 nM BK, or with 1 ng/ml EGF were immunoprecipitated with anti-α5β1 integrin antibody as described in Methods. Immunoblotting was performed with antibodies against EGFR (Panel A) and BK B_2 receptor (Panel B). The blots shown are representative of four experiments.

A. Co-immunoprecipitation experiments show that α5β1 integrin and EGFR co-immunoprecipitate and that their association can be increased by stimulation of mIMCD-3 cells with 100 nM BK, but not with EGF. Inset presents representative Western blot with antibody against EGFR showing immunoreactive band at 175 kDa. Blot was stripped and re-probed with antibody against α5 integrin to control for immunoprecipitation and protein loading. Immunoreactive band at 150 kDa is shown.

B. BK B_2 receptor co-immunoprecipitates with α5β1 integrin. Inset presents representative Western blot with antibody against BK B_2 receptor showing immunoreactive duplet at 42/40 kDa. Blot was stripped and re-probed with antibody

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against α5 integrin to control for immunoprecipitation and protein loading. Immunoreactive band at 150 kDa is shown.

IP, immunoprecipitation; IB, immunoblot

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Table: **mIMCD-3 cells express mRNA for α and β integrin subunits**

The expression of mRNA was assessed by the Oligo GEArray as described in Methods. Proteins message for which we were able to detect are shown in bold.

	Gene Symbol	*Position*	*Signal Intensity*
1	**α2**	44	+
2	**α2b**	45	++++
3	**α3**	46	+++
4	α4	47	–
5	α5	48	_
6	**α6**	49	+
7	**α7**	50	+
8	α8	51	–
9	αE	52	–
10	αL	53	–

	Gene Symbol	*Position*	*Signal Intensity*
11	αM	54	–
12	**αV**	55	+++
13	**αX**	56	++
14	**β1**	57	++++
15	β2	58	–
16	β3	59	–
17	β4	60	–
18	β5	61	–
19	**β6**	62	++
20	**β7**	63	++

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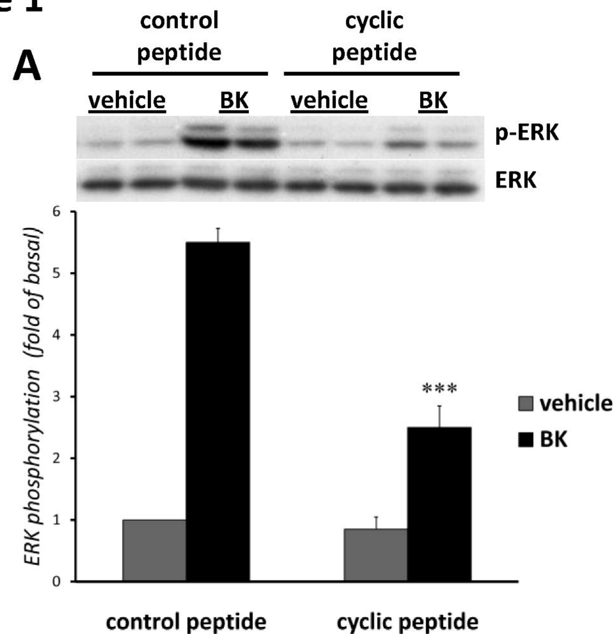
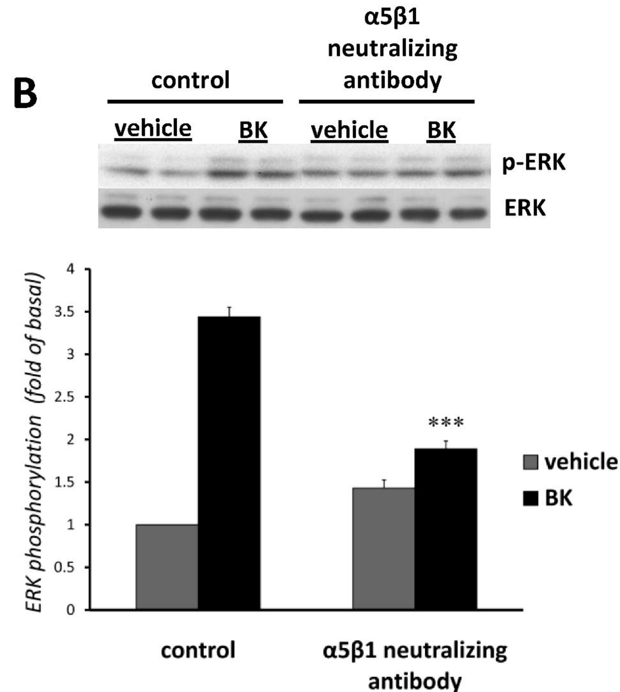
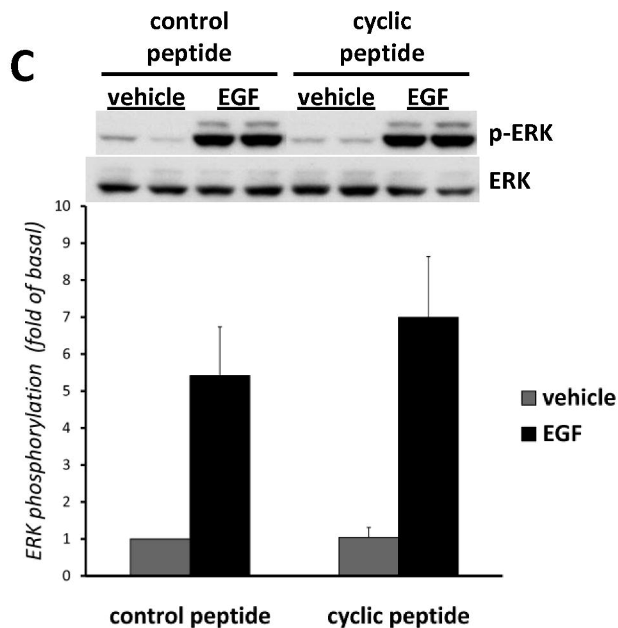
Figure 1







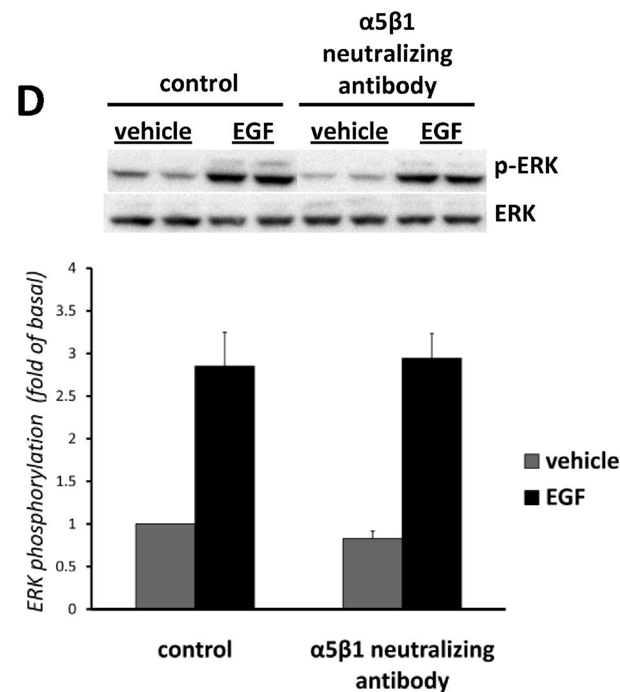


Figure 2

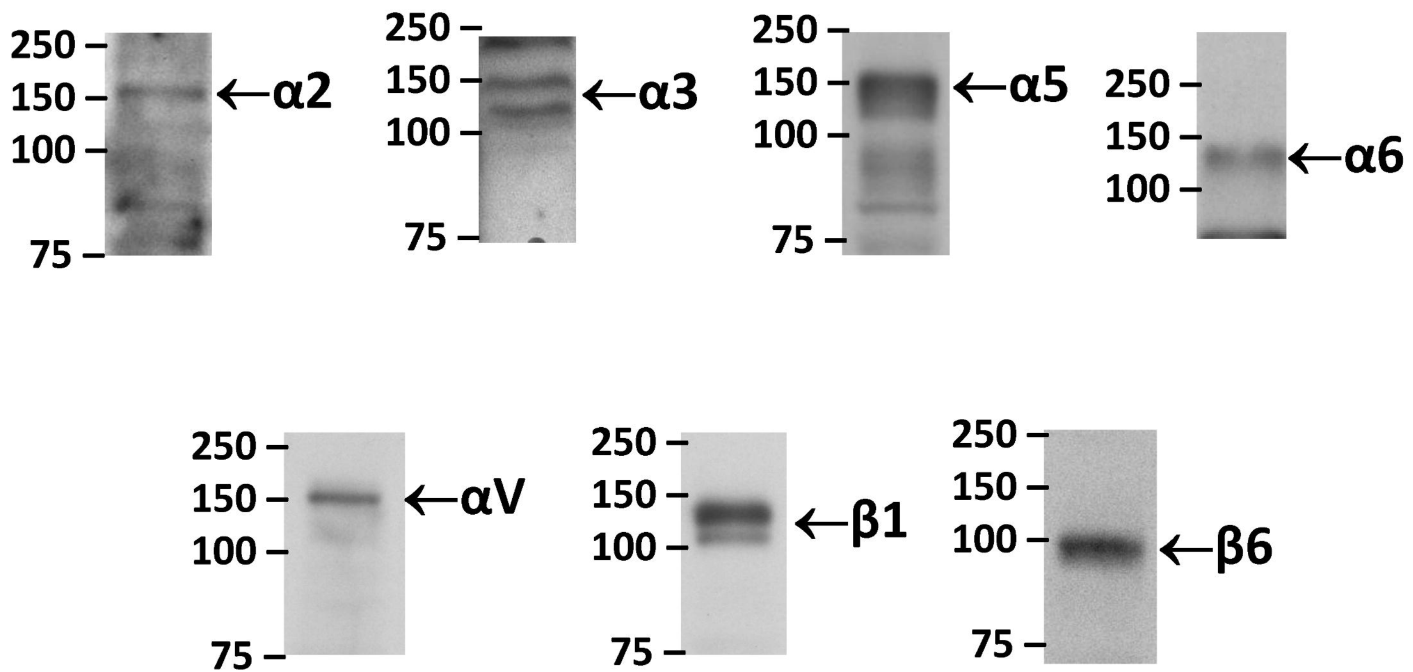
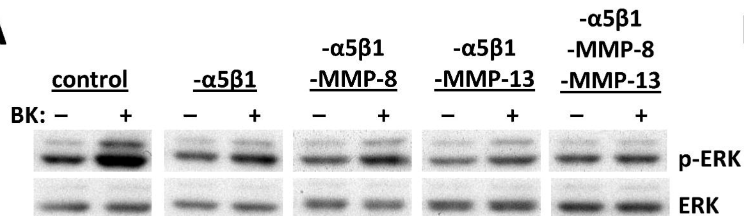
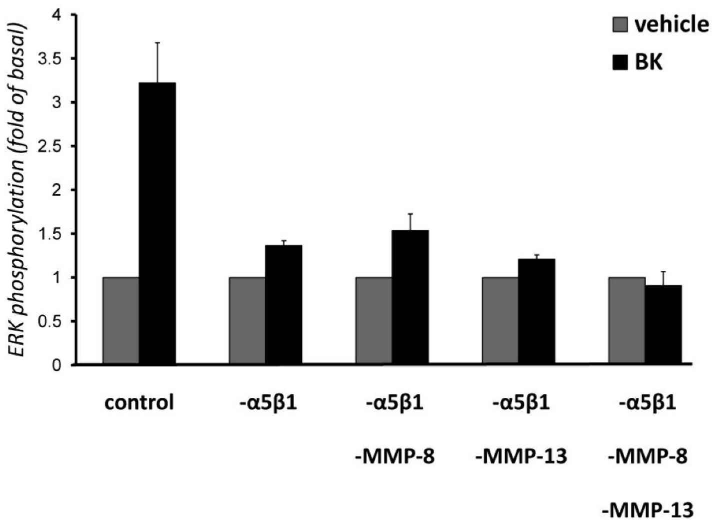
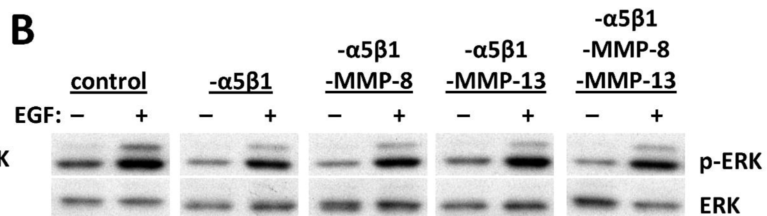


Figure 3







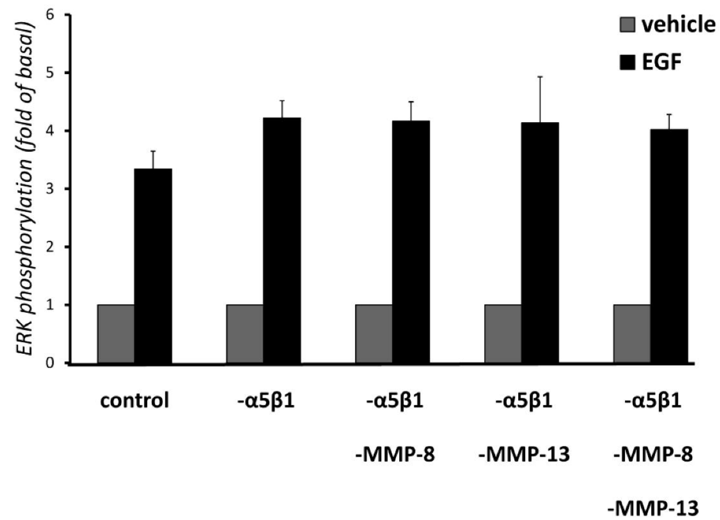
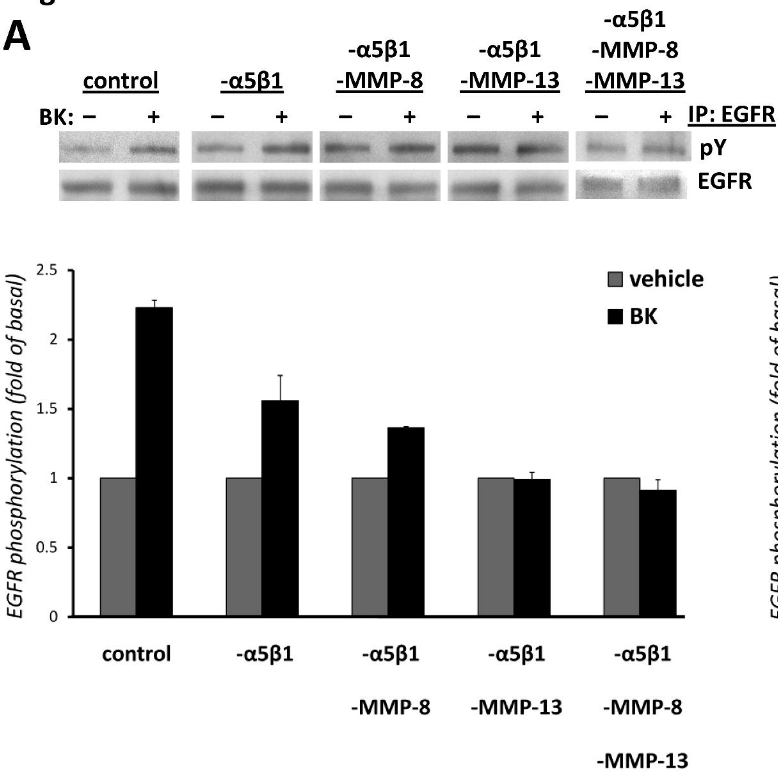
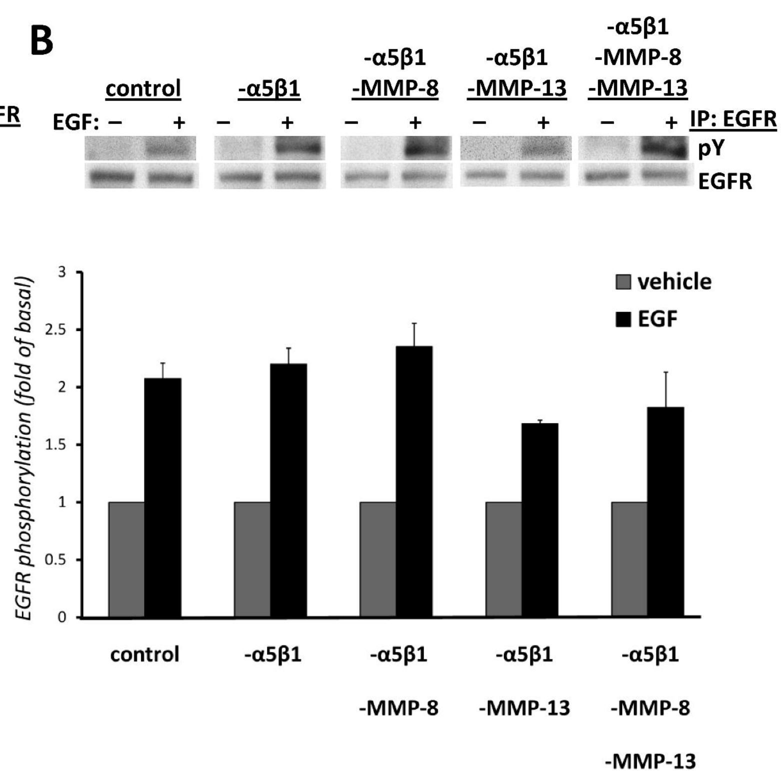


Figure 4





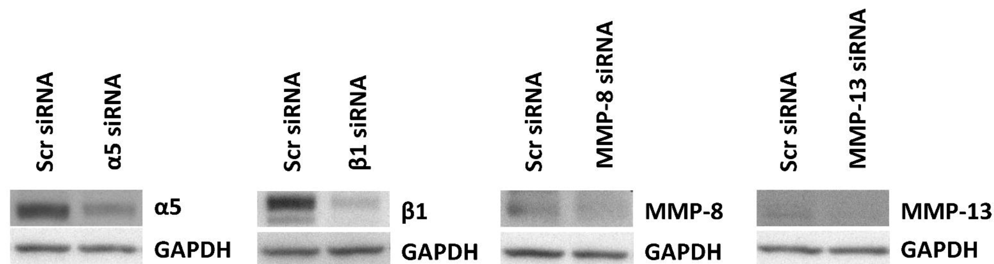


Figure 5