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Bmx is a downstream Rap1 effector in VEGF-induced endothelial cell activation

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Abstract

We had previously shown that Rap1 mediates certain of the signaling pathways involved in VEGF-induced endothelial cell migration, although the downstream Rap1 effectors are not known. Towards the goal of identifying those effectors, we utilized a commercially available antibody array filter to identify proteins that either directly interact with Rap1 or interact indirectly through a multi-protein complex. The protocol identified 10 possible Rap1-interacting proteins, including the Bmx non-receptor tyrosine kinase. The conclusion that VEGF treatment leads to a Rap1/Bmx complex was confirmed by an experiment in which cell lysates from VEGF and control cells were immunoprecipitated with Bmx antibodies and Western blotting was done using anti-Rap1 antibodies. VEGF treatment led to the recruitment of Bmx to the CAS scaffolding protein, and inhibition of the Bmx kinase blocked VEGF-induced cell migration. Formation of a Rap1/Bmx complex was not observed in cells transfected with an expression vector for a dominant-negative Rap1, indicating that Bmx is a downstream Rap1 effector in VEGF-induced endothelial cell activation.

Keywords: VEGF; Rap1; Bmx; Endothelial cells; Angiogenesis

The growth of new blood capillaries from existing vessels is essential for embryonic development, tissue regeneration, and remodeling [1,2]. Angiogenesis also contributes to pathologic conditions including tumor growth and metastasis [3,4]. Angiogenesis is a multistep process involving degradation of extracellular matrix, endothelial cell migration and proliferation, and tube formation. Much progress has been made in the identification of extracellular activators of endothelial cell function, as well as their cell surface receptors. Growth factors, acting in a paracrine manner [5,6], as well as adhesion molecules that recognize extracellular matrix molecules [7] regulate endothelial cell function. Less certain are the signaling pathways mediating the cellular responses to these activators.

Vascular endothelial growth factor (VEGF) is a critical pro-angiogenic factor [5] that has received attention as a target for angiogenic inhibition for several reasons including the observations that blocking VEGF's actions inhibits the growth of tumors in animal models [8,9]. VEGF stimulates multiple endothelial cell activities through binding to two distinct receptor tyrosine kinases, Flt1 (VEGFR1) [10,11] and KDR (FLK1, VEGFR2) [12,13]. Although expression of both receptors occurs in endothelial cells, KDR and not Flt1 is able to mediate the mitogenic and chemotactic effects of VEGF [14].

Our laboratory had previously shown that the small GTPase Rap1 plays a necessary role in the signaling mechanism by which VEGF regulates endothelial cell adhesion to the extracellular matrix [15]. Expression of a dominant-negative Rap1 in human umbilical vein endothelial (HUVE) cells blocks both VEGF-induced focal adhesion turnover as well as integrin activation. The molecular interactions that mediate VEGF-induced Rap1 activation involve the recruitment to activated

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receptor of a complex containing the Crk adaptor protein with the C3G guanine exchange factor, followed by binding of the complex to the CAS scaffolding protein; C3G then activates Rap1. It remained uncertain from this earlier study as to the signaling pathways downstream from Rap1 that facilitate the VEGF-induced cellular responses.

Several other earlier studies have documented a role for Rap1 in growth factor and integrin-mediated cell adhesion in a variety of cell types [16–18]. For example, Rap1b modulates the activation of integrin αIIβ3 and the actin cytoskeleton in megakaryocytes [19]. Also, mouse embryonic fibroblasts deficient in C3G exhibit defective integrin-dependent adhesion, and the phenotypic alteration is correctable by expression of a constitutively active Rap1 mutant [17]. Rap1 also appears to be involved in the formation of cell-to-cell adheren junctions, as it has been observed that Rap1-mutant Drosophilae cell clones cluster their adheren junctions to one side of the cells resulting in the dispersion of mutant cell clones into surrounding wild type tissue [18]. While it is not certain whether Rap1 mediates similar cellular activities in mammalian cells, it is noteworthy that the adheren junction protein AF-6 binds Rap1 with high affinity [20].

There is no clear consensus as to the downstream effectors that mediate Rap1's biological activities, and so it is not known whether Rap1's effects on integrin activation, as well as other Rap1's putative activities, are due to effects on focal adhesions, the actin cytoskeleton, adheren junctions, or through multiple effectors. The goal of the current study was to clarify cell-signaling pathways that are downstream from Rap1 in VEGF-induced cellular activation. Our strategy was to identify signaling proteins that complex with Rap1 in a VEGF-dependent manner taking advantage of a commercially available antibody array that contains on a single filter antibodies of 400 cell-signaling proteins arranged in a grid. This strategy allows for the identification of proteins that either directly interact with a target protein or interact indirectly as being contained in a multi-protein complex.

Materials and methods

Cell culture. Human umbilical vein endothelial (HUVE) cells were isolated as previously reported [21]. Passage two cells were cultured on 0.2% gelatin-coated tissue culture plates in M199 containing 20% newborn calf serum, 5% human serum, and endothelial growth factor (Sigma), 7.5 μg/ml.

Reagents and antibodies. Anti-Bmx monoclonal antibodies were from Transduction Laboratories. Polyclonal anti-Rap1, anti-p130CAS, and anti-Fas antibodies were from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugation of anti-Rap1 antibody was performed using the EZ-link Plus (Pierce) kit as recommended by the vendor. Peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse immunoglobulins were from Amersham–Pharmacia Biotech. The 165 amino acid isoform of human VEGF was purchased from R&D Systems. LFM-A13 was from BioMol. The expression plasmid encoding dominant-negative Rap1 (Rap(N17)) has been described previously [22].

Screening of Antibody-Array. HUVE cells, grown to subconfluence on 2 × 10 cm dishes, were serum starved for 6 h and incubated with or without 50 ng/ml VEGF for 10 min. All subsequent steps were performed at 4 °C. The cells were washed once with PBS, scraped from the dishes and centrifuged, and suspended in 4 ml lysis buffer (10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.2 mM Na_3VO_4, 30 mM $Na_4P_2O_7$, 30 mM NaF, 1mM EDTA, 1 mg/ml leupeptin, 0.7 mg/ml pepstatin A, and 1% Triton X-100). The cell extracts were incubated for 16 h at 4 °C with the Antibody-Array filter (Hypromatrix) that had previously been incubated in blocking solution consisting of 5% dried milk in TBS-T (150 mM NaCl, 25 mM Tris, and 0.05% Tween 20, pH 7.5) for 1 h at room temperature. The Antibody-Array filter was then washed 3 × 15 min with TBS-T. The filters were incubated with HRP-conjugated anti-Rap1 antibody in TBS-T for 4 h at room temperature and then washed 4 × 15 min with TBS-T. The reactions were visualized through enhanced chemiluminescence (Pierce).

Western blotting. HUVE cells were grown on 6 cm dishes until subconfluent. The cells were incubated in serum-free DMEM for 6 h and VEGF (50 ng/ml) was added to certain wells for 10 min at 37 °C. Cells were then lysed and primary antibodies were added. After a 1 h incubation at 4 °C, the antibodies were immobilized on protein A–Sepharose beads (Sigma), the beads were washed three times with lysis buffer and boiled in 40 μl SDS–PAGE sample-loading buffer. Proteins were separated on SDS–PAGE gels, transferred to nitrocellulose (Bio-Rad), and blotted with appropriate antibodies. The filters were blocked in TBS-T plus 5% dried milk at 4 °C overnight and probed with specific antibodies for 2 h at room temperature. After washing, the filter was incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG, and reactions were visualized through enhanced chemiluminescence (Pierce).

Cell migration assay. The migration assay utilized a modified Boyden chamber and was performed essentially as previously described [23]. HUVE cells were grown to 50% confluency, lifted from the dish using non-enzymatic cell dissociation solution (Sigma), centrifuged for 2 min at 1000 rpm, and resuspended in M199 containing 0.5% BSA. The cells were plated on fibronectin-precoated transwells (Costar) at a density of 10^5 cells/well. M199 with VEGF (10 ng/ml) was used as a chemoattractant in the lower wells. Cell migration assays were performed for 8 h at 37 °C in a CO_2 incubator. Cells that had migrated through the transwells were stained with CMFDA (Sigma) dye. Four different fields were recorded with a digital camera for samples performed in triplicate, and the results are representative of three independent experiments.

Results and discussion

A commercially available antibody array was utilized to identify cellular proteins that complex to Rap1 in a VEGF-dependent manner. The array consists of antibodies to 400 cell-signaling proteins placed as a grid on a nitrocellulose filter. The rationale for using the array is that Rap1 will localize to the position on the filter containing an antibody to a protein it complexes with, and Western blotting using anti-Rap1 antibody will identify the relevant location on the grid, and thus the identity of Rap1's protein partner. For the experimental results shown in Fig. 1, serum starved HUVE cells were treated with or without VEGF for 10 min, cell lysates were prepared and incubated with

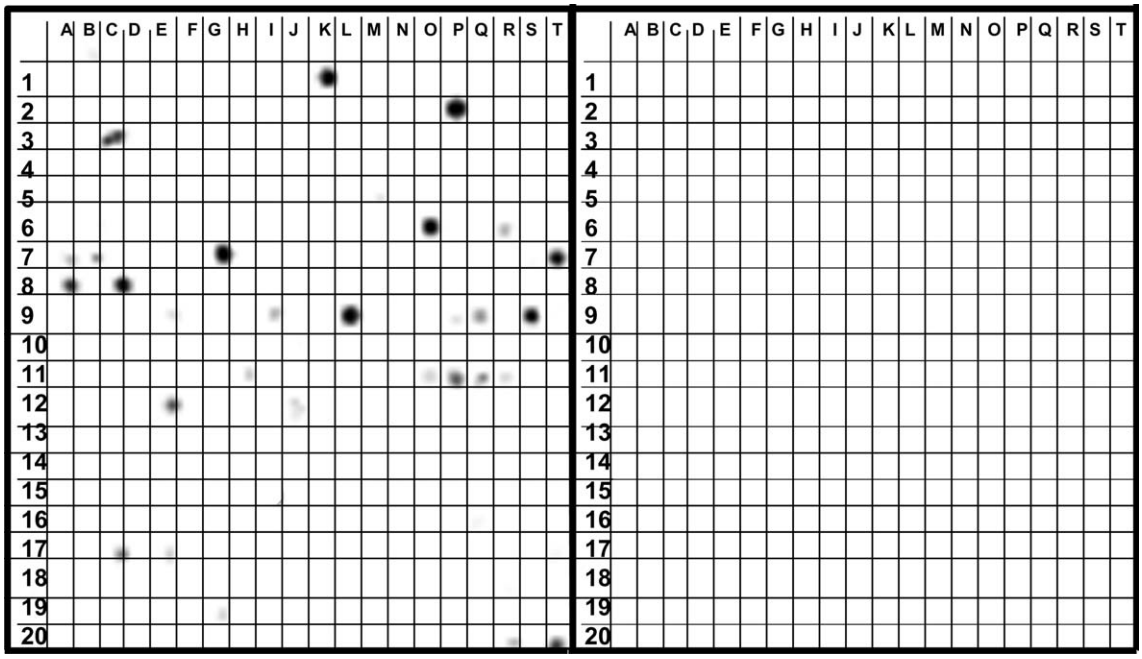

Fig. 1. Screening of Antibody-Array filter. Serum starved HUVE cells were treated with VEGF (left blot) or left untreated (right blot). Cell lysates were incubated with the filters; the filters were washed and then incubated with HRP-conjugated anti-Rap1 antibody.

the nitrocellulose filter. Western blotting was performed using horseradish peroxidase-conjugated anti-Rap1 antibody.

The results of Fig. 1 demonstrate that Rap1 interacted with 10 cell-signaling proteins in a VEGF-dependent manner. The 10 interacting proteins participate in a diverse set of cellular activities (Table 1) with one of these (Bmx) known to play necessary role in cellular activities involved in cell adhesion, migration, and survival [24,25]. Six (Bin1 [26], DcR1 [27], FAF-1 [28], Fas [29], FADD [29], and Hsp-70 [30]) of the identified proteins participate in cellular activities related to cell survival or apoptosis. Three (HDAC1, Brm, and Elongin A) are nuclear proteins [30–32]. HDAC1 [31] and Brm [32] function in the regulation of chromatin-remodeling and subsequent transcriptional activity. Elongin A [33] promotes efficient RNA elongation by RNA polymerase II.

Our subsequent experiments focused on Bmx because earlier studies have indicated that this non-receptor tyrosine kinase is expressed at high levels in endothelial cells, and participates in the cell-signaling pathways activated by multiple extracellular activators. We validated the conclusion that Bmx complexes with activated Rap1 by treating serum starved HUVE cells with or without VEGF, preparing cell lysates, and immunoprecipitating with anti-Bmx antibodies. Western blotting using anti-Rap1 antibodies demonstrated Rap1/Bmx complex formation in VEGF-treated but not untreated cells. Incorporation of Bmx into the Rap1 protein complex was dependent upon Rap1 activation as no complex was formed in cells trans-

Table 1
Results of the antibody array screening protocol

Position	Protein	Protein characteristics
K1	Bin1	c-Myc-interacting protein, pro-apoptotic
P2	BMX	Member, Tec family of non-receptor tyrosine kinases
C3	Brm	Subunit of SW-SNF chromatin-modeling complex
O6	DcR1	Decoy receptor for death ligands, pro-survival
G7	ElonginA	Transcription corepressor
T7	FADD	FAS-associated death domain
A8	FAF-1	Fas-associated factor-1, pro-apoptotic
C8	Fas	Death-inducing receptor for FAS-L, pro-apoptotic
L9	HDAC1	Histone deacetylase 1
S9	HSP-70	Heat shock protein-70, pro-survival

The proteins corresponding to the filter (Fig. 1) positions (column 1) showing a positive signal were identified from the Hypromatrix user manual and are indicated in the second column. The third column gives a brief description as to the protein's cellular function.

fected with an expression vector for dominant-negative Rap1.

The antibody array screen indicated that FAS, a death-inducing receptor for FAS-L, complexes with Rap1 in a VEGF-dependent manner. The experimental results shown in Fig. 2C add supporting evidence to this conclusion, as it demonstrates that VEGF treatment stimulates an increased amount of FAS in anti-Bmx antibody derived immunoprecipitates of cell extracts.

We then tested whether VEGF treatment leads to a recruitment of Bmx to the CAS scaffolding protein. The rationale for this experiment was twofold. First, Bmx is recruited to CAS upon integrin activation [24], and there are a number of parallels between integrin and growth factor induced signaling cascades. Second, studies [34] have shown that growth factors activate the upstream Rap1 effector C3G by facilitating its recruitment from the cell cytoplasm to CAS. We previously showed that VEGF treatment leads to the recruitment of a C3G–Crk protein complex to CAS, and this is required for Rap1 activation [15]. The results shown in Fig. 2E indicate that VEGF treatment does lead to the formation of a Bmx–Cas protein complex.

We had previously reported [23] that a dominant-negative Rap1 blocks VEGF-induced integrin activation, demonstrating that Rap1 mediates certain of the signaling pathways involved in VEGF-induced cell migration. To determine whether Bmx participates in the Rap1-mediated response, we tested the consequence of Bmx inhibition on VEGF-induced cell migration using a Boyden chamber. LFM-A13, a specific inhibitor of the Bruton's tyrosine kinases [35,36], was utilized to block Bmx catalytic activity, and Fig. 3 shows that LMF-A13 treatment blocked VEGF-induced cell migration.

Bmx (bone marrow tyrosine kinase), also known as Etk (epithelial and endothelial tyrosine kinase), is a member of the Btk (Bruton's tyrosine kinase) family of non-receptor tyrosine kinases [37]. Bmx and two other members of this family (Btk, Itk, and Tec) share a common domain structure including a pleckstrin homology domain, a Src homology 3 (SH3) domain, a SH2 domain, and a catalytic tyrosine kinase domain [38]. Each kinase

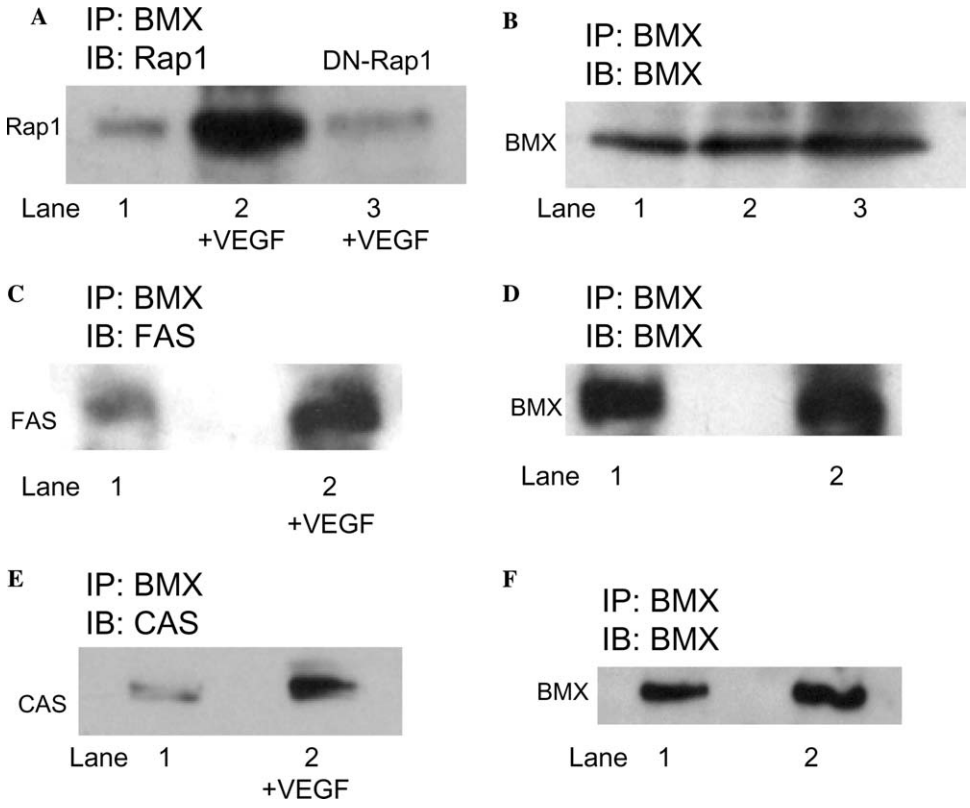

Fig. 2. VEGF treatment leads to the recruitment of Bmx to a Rap1-containing complex. (A) HUVE cells were plated at 50% confluence on 6 cm dishes, and either mock transfected (lanes 1 and 2) or transfected with the DN-Rap1 expression vector (lane 3). After 48 h, the cells were serum starved and then incubated with (lanes 2 and 3) or without (lane 1) VEGF for 10 min. The cells were lysed and immunoprecipitation was done using anti-Bmx antibodies, and Western blotting was done using anti-Rap1. (B) The blot used in (A) was stripped and reprobed using anti-Bmx antibody in order to ensure equal loading of samples. (C,E) Cells were plated at 90% confluence, serum starved, and VEGF treatment was for 10 min. Cell lysates were immunoprecipitated using anti-Bmx antibodies, and Western blotting was done using anti-Fas (C) or anti-Cas (E) antibodies. The blots used for (C,E) were stripped and reprobed using anti-Bmx antibodies (D,F).

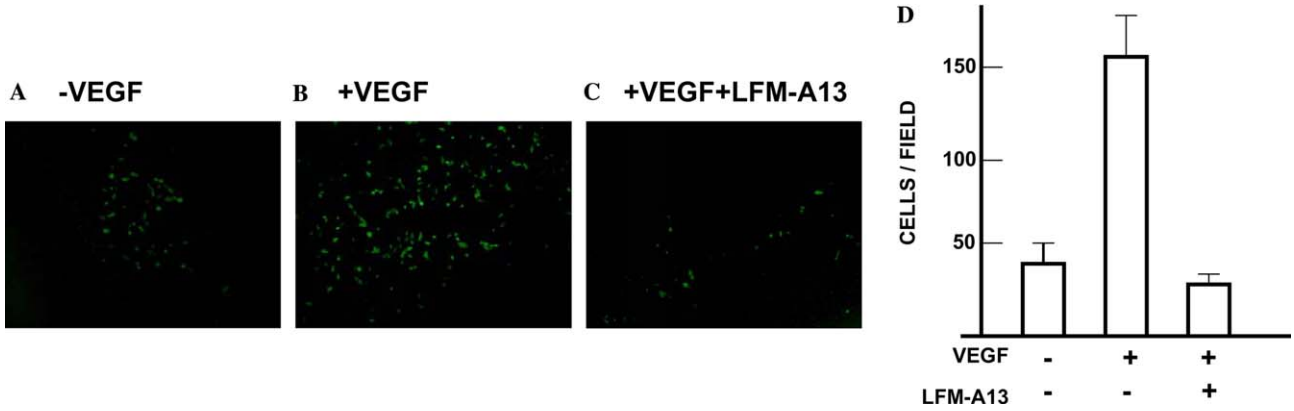

Fig. 3. Bmx inhibition blocks VEGF-induced cell migration. HUVE cells were plated on fibronectin-precoated transwells (Costar) at a density of 10^5 cells/well. LFM-A13, 25 μM, was added to the cells shown in (C). M199 with VEGF (10 ng/ml) was used as a chemoattractant in the lower wells (B,C). Cell migration assays were performed for 8 h at 37 °C in a CO_2 incubator. Cells that had migrated through the transwells were stained with CMFDA dye and visualized under a fluorescent microscope. The results shown in (A)–(C) are representative of four different fields recorded for each well, and each experimental condition was performed in triplicate. Quantification of the experimental results is given in (D).

has a unique expression pattern, and Bmx is expressed in endothelial cells, epithelial cells, and monocytes/macrophages including prostate tissues [39,40].

Bmx appears to mediate two cellular activities, cell migration and cell survival. In regard to cell migration, it was shown that for Bmx-transfected COS-7 and HEK293 cells Bmx is activated in response to cell adhesion on fibronectin, and Bmx activation is required for enhanced cell migration on the substrate [24]. Of particular significance to our study, integrin-mediated Bmx activation is dependent upon its recruitment to tyrosine phosphorylated CAS. CAS is both an upstream and downstream Bmx effector in integrin-mediated signaling, as CAS is a substrate for the Bmx kinase. Bmx-mediated cell migration has also been demonstrated in HUVE cells [41]; this study examined signaling mechanisms activated in response to tumor necrosis factor. It was shown that a constitutively active form of Bmx enhanced, whereas a dominant-negative Bmx blocked, TNF-induced cell migration and tube formation.

Several lines of evidence have implicated Bmx in the apoptosis pathway. Interestingly, Bmx has been reported to be able to induce both pro- as well as anti-apoptotic signals. Overexpression of Bmx protects prostate cancer cells from apoptosis induced by photodynamic therapy or thapsigargin [40]; on the other hand, Bmx sensitizes a mast cell line toward apoptosis in response to C-CSF [42]. These seemingly contradictory results might be explained by a more recent study documenting that caspase-mediated cleavage converts Bmx to a pro-apoptotic mediator [25]. Other members of the Btk family also mediate anti-apoptotic signaling pathways. Of particular significance to our study, Btk was shown to directly associate with Fas and block the death receptor's signaling [43].

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