Regulation of G Protein-Coupled Receptor Activities by the Platelet-Endothelial Cell Adhesion Molecule, PECAM-1[†]

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ABSTRACT: It is becoming increasingly evident that the cell−cell junction is a major signaling center. Here we show that the Gαq/11 subunit of heterotrimeric G proteins forms a complex with platelet-endothelial cell adhesion molecule 1 (PECAM-1), a junctional protein that has been shown to be involved in mechanosignaling in endothelial cells. To understand the role of PECAM-1 in this complex, we determined the critical regions of PECAM-1 involved in this interaction. By expressing truncated forms of PECAM-1 in human embryonic kidney (HEK293) cells, we found that the cytoplasmic domain of PECAM-1 is not required for its association with Gαq/11. Domain swapping of PECAM-1 with intracellular cell adhesion molecule 1 (ICAM-1), a protein that does not form a complex with Gαq/11, provides evidence that the extracellular domain of PECAM-1 is critical for this interaction. This result also suggests that PECAM-1 does not directly interact with Gαq/11. Coexpression of bradykinin receptor B2 (BKRB2), a Gαq/11-coupled receptor, with PECAM-1 enhances formation of the PECAM-1−Gαq/11 complex, suggesting an interaction between PECAM-1 and BKRB2. Co-immunoprecipitation experiments indicate that these two molecules indeed form a complex when expressed in HEK293 cells. Activation of ERK1/2 by bradykinin in HUVEC is enhanced when PECAM-1 expression is inhibited by transfection of small interference RNA against PECAM-1. Taken together, our results provide evidence of interaction of PECAM-1 with BKRB2 and of its possible role in regulating G protein-coupled receptor (GPCR) and G protein functions.

Platelet-endothelial adhesion molecule-1 (PECAM-1 or CD31)[1] is a 130 kDa glycoprotein abundantly expressed in endothelial cells. PECAM-1 is expressed less abundantly in platelets, monocytes, neutrophils, and subsets of T- and B-lymphocytes (*1*). PECAM-1 belongs to the immunoglobulin (Ig)-like adhesion molecule superfamily and consists of an extracellular domain comprised of six Ig-like homology domains, a transmembrane domain, and a cytoplasmic domain. In endothelial cells, PECAM-1 is primarily located at the cell−cell junctions through homophilic interaction via the first and second Ig domains (*2–5*). The discovery of two conserved immunoreceptor tyrosine-based inhibitory motifs (ITIMs) at the cytoplasmic domain (*6*) has enabled investigators to reveal the roles of PECAM-1 in regulating signal transduction pathways. Tyrosine phosphorylation at tyrosine residues 663 and 686 within the ITIM domains has been demonstrated to be crucial in its interactions with different signaling molecules, especially those containing SH2 domains, such as SHP1, SHP2, SHIP, and PLC-γ1 (*7, 8*). An ITIM-independent function of PECAM-1 has been proposed to serve as a scaffolding protein in regulating protein phosphorylation by recruiting an enzyme (kinase or phosphatase) and a protein substrate into the proximity of each other (*9*). For example, PECAM-1 has been shown to regulate phosphorylation states of two junctional proteins, β- and γ-catenins, through the interaction with its non-ITIM region of the cytoplasmic tail (*2, 10–12*). Two members of the STAT (signal transducer and activator of transcription) family, STAT3 and STAT5a, have also been shown to belong to this class of PECAM-1 interacting proteins (*9, 13*).

An additional role of PECAM-1 in mediating the mechanosignaling pathway has emerged (*14–16*). It has been shown that PECAM-1 mediates the rapid response of endothelial cells to the rate change of fluid shear stress (*17–19*). Recently, PECAM-1, together with VE-cadherin and VEGFR2, has been shown to form a mechanosensory complex (*19*). All this evidence indicates that PECAM-1 plays a crucial role in the early events of the mechanosensory process. Previously, we have demonstrated that heterotrimeric G protein α subunit q/11 (Gαq/11) is involved in an early event in the mechanosignaling pathway as it becomes activated within seconds of fluid shear stress stimulation (*20, 21*). These observations prompted us to examine whether there is a link between these two molecules. Indeed, we observed a PECAM-1-dependent colocalization of Gαq/11 at the cell−cell junction in endothelial cells (manuscript submitted). Here, we provide direct molecular evidence of PECAM-1 forming a complex with Gαq/11 and a Gαq/11-coupled receptor,

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[1] Abbreviations: PECAM-1, platelet-endothelial cell adhesion molecule-1; ICAM-1, intracellular cell adhesion molecule-1; G protein, guanine nucleotide binding protein; GPCR, G protein-coupled receptor; HUVEC, human umbilical cord vein cells; BK, bradykinin; BKRB2, bradykinin receptor B2; HOS, hyperosmotic shock; ERK1/2, extracellular signal-regulated kinase 1/2.

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bradykinin receptor B2 (BKRB2). We also mapped the domains of PECAM-1 required for this interaction. Furthermore, we provide evidence that PECAM-1 regulates BKRB2 activity through this interaction.

MATERIALS AND METHODS

Reagents. Bradykinin was obtained from EMD Bioscience. Anti-FLAG monoclonal antibody (M2) and agarose were obtained from Sigma. Antibodies against PECAM-1, BKRB2, and Gαq/11 were obtained from Santa Cruz. Anti-ERK1/2 and phospho-ERK1/2 antibodies were purchased from Cell Signaling. Rabbit antiserum against the PECAM-1 cytoplasmic domain was raised by immunizing rabbits with the purified PECAM-1 cytoplasmic domain (generated by PreScission protease cleavage of a recombinant GST−PECAM-1 cytoplasmic domain fusion protein) (*18*). siRNA against human PECAM-1 [sense sequence, r(GGGACAUAUAAAUGUACUG)dTdT] and negative control siRNA were purchased from Ambion.

Cells. HEK293-H cells were obtained from Invitrogen. Primary HUVEC were harvested as described previously (*22*). Bovine aortic endothelial cells (BAEC) were obtained from Cambrex.

Vector Construction. All mammalian expression constructs were produced in pcDNA3.1/hygromycin (pcDNA3.1/Hyg) (Invitrogen) unless otherwise specified. Human PECAM-1 cDNA (GenBank accession number NM_000442) was amplified by polymerase chain reaction (PCR) from human brain QUICK-Clone cDNA (Clontech) using the following primers: CD31-1, 5′-TCTCAGGATGCAGCCGAGGTGGGCCCAA-3′; and CD31-2AS, 5′-CTAAGTTCCATCAAGGGAGCCTTCCGTTC-3′. Human Gαq cDNA (GenBank accession number NM_002072) was amplified by PCR from human brain QUICK-Clone cDNA (Clontech) using the following primers: 5′-ATGACTCTGGAGTCCATCATGGCGTGC-3′ and 5′-TTAGACCAGATTGTACTCCTTCAGGTTCAA-3′. The amplified PCR products were subcloned into pCR2.1 using a TOPO PCR cloning kit (Invitrogen) and were confirmed by DNA sequencing. The expression vector of Gαq was generated by excising Gαq cDNA from pCR2.1 by restriction enzyme digestion, and the released fragment was cloned into pcDNA3.1/Hyg. The vector containing PECAM-1 cDNA was used as a template to generate expression constructs for epitope-tagged wild-type and mutant PECAM-1. The sequences of the primer pair used for PCR to generate the FLAG-tagged PECAM-1 were 5′-C C C *A A G C T T A* C C A T G C A G C C G A G G T G G G C-CCAAGGGGCCACG-3′ (*Hin*dIII-CD31-5′ forward primer) and 5′-GCG*GTCGAC*CTACTTATCGTCGTCATCCTTGTAATCAGTTCCATCAAGGGAGCCTTCC-3′ (*Sal*I-CD31-FLAG-3′ AS reverse primer), with *Hin*dIII and *Sal*I sites in italics, respectively. The PCR products were digested with *Hin*dIII and *Sal*I and ligated to *Hin*dIII- and XhoI-digested pcDNA3.1/Hyg. The encoded protein has a FLAG epitope tag (amino acid sequence DYKDDDDK) linked to the carboxyl terminus of PECAM-1.

FLAG-tagged cytoplasmic deletion mutant constructs were generated according to their exon structure (*8*). Reverse primers were designed according the ending sequences of each exon with an additional *Age*I site to facilitate the cloning. Vector containing FLAG-tagged PECAM-1-ΔEX_{15-16} (lacking exons 15 and 16) was first constructed, and the insert was removed by *Hin*dIII and *Age*I digestion to generate a vector containing a FLAG tag sequence flanked by an *Age*I site (encoding two additional amino acids, TG). This vector was designated as pcDNA3.1/Hyg/C-FLAG and was used for subsequent construction of C-terminally FLAG-tagged protein expression vectors. The primers used for ΔEX_{15-16} construction were *Hin*dIII-CD31-5′ and *Kpn*I-CD31-EX14ST-FLAG-R [5′-GG*GGTACC*CTACTTATCGTCGTCATCCTTGTAATCACCGGTAGGGACAGCTTTCCGGACTTCACTG-3′ (*Kpn*I site in italics)]. The PCR products were digested with *Hin*dIII and *Kpn*I and were ligated to *Hin*dIII- and *Kpn*I-digested pcDNA3.1/Hyg. Other deletion mutants were generated by PCR using forward primer CD31-3 (5′-GGAATCTTCCTTCACACATCTGGACCAAGG-3′) and the following corresponding reverse primers: PECAM-1-ΔEX_{11-16}, CD31-EX10ST-R, 5′-C*ACCGGT*CCTGGACATTTCCACTGGCATCTG-3′; PECAM-1-ΔEX_{12-16}, CD31-EX11ST-R, 5′-C*ACCGGT*GTAATGACTGTTAGCTTCCATATT-3′; PECAM-1-ΔEX_{13-16}, CD31-EX12ST-R,5′-C*ACCGGT*TTTATTATCATTTATTGGTTTCAT-3′; and PECAM-1-ΔEX_{14-16}, CD31-EX13ST-R, 5′-C*ACCGGT*TTTGTGAGACTCAGCTGAGGACAC-3′.

*Aat*II-digested (digested between nucleotides 1410 and 1411) and *Age*I-digested PCR products along with *Hin*dIII- and *Aat*II-digested PECAM-1 1.4 kb fragment (released from pcDNA3.1/Hyg/PECAM-1-FLAG) were ligated to the *Hin*dIII- and *Age*I-digested pcDNA3.1/Hyg/C-FLAG vector.

A PECAM-1 cytoplasmic deletion mutant was generated with the following reverse primer: CD31-DE-CD-R-*Age*I, 5′-C*ACCGGT*TTTGGCCGCAATGATCAAGAGAG-3′.

cDNA encoding full-length ICAM-1 was obtained from Invitrogen. FLAG-tagged ICAM-1 was produced by PCR using the following primers: *Hin*dIII-ICAM1-5′, 5′-CC*AAGCTT*ACCATGGCTCCCAGCAGCCCC-3′; and ICAM1-3′end-*Age*I-AS, 5′-GT*ACCGGT*GGGAGGCGTGGCTTGTGTG-3′. The PCR products were digested with *Hin*dIII and *Age*I and were ligated into the *Hin*dIII- and *Age*I-digested pcDNA3.1/Hyg/C-FLAG vector.

To generate PECAM1−ICAM1 hybrid molecules and PECAM-1 Ig domain deletion mutants, overlap extension PCR (PCR sewing) (*23*) was used. pcDNA3.1/Hyg/PECAM-1-FLAG and pcDNA3.1/Hyg/ICAM-1-FLAG were used as templates to generate PCR products for the corresponding domains. For PECAM1−ICAM1 hybrid vector construction, primers were designed with overhanging sequences at 3′-end for subsequent overlap extension PCR. In the primers that were designed, P stands for PECAM-1 and I stands for ICAM-1, ext represents the extracellular domain, tm represents the transmembrane domain, and cyt represents the cytoplasmic domain. R and F indicate the orientation (reverse and forward, respectively) of each primer. Primers *Hin*dIII-CD31-5′ and CD31-3′end-*Age*I-R (5′-AATACCGGTAGTTCCATCAAGGGAGCCTTC-3′) were used in conjunction with proper primers in PCRs involved in amplifying PECAM-1 extracellular and cytoplasmic domains. Primers *Hin*dIII-ICAM1-5′ and ICAM1-3′end-*Age*I-R (5′-AATACCGGTAGTTCCATCAAGGGAGCCTTC-3′) were used in conjunction with proper primers in PCRs involved in amplifying ICAM-1 extracellular and cytoplasmic domains: Ptm-Icyt-R,5′-CCGCTGGCGGTTCAGAAAATAACATTTGGC-3′; Ptm-Icyt-F, 5′-TGTTATTTTCTGAACCGCCAGCG-

GAAGATC-3′; Itm-Pcyt-R, 5′-CTTGGCTTTCCTATAGAG-GTACGTGCTGAG-3′; Itm-Pcyt-F, 5′-ACGTACCTCTA-TAGGAAAGCCAAGGCCAAG-3′; Pext-Itm-R, 5′-GAT-GATGACAATTCCTTTCTTCCATGGGGC-3′; Pext-Itm-F, 5′-TGGAAGAAAGGAATTGTCATCATCACTGTG-3′; Iext-Ptm-R, 5′-CACTGCAATAAGCTCATACCGGGGGGA-GAG-3′; and Iext-Ptm-F, 5′-CCCCGGTATGAGCTTATTGC-AGTGGTTATC-3′. PCR fragments were generated using proper templates and primers and were sewed by their overlapping sequences on the primers. The restriction enzyme-digested (*Hin*dIII and *Age*I) PCR products were then cloned into pcDNA3.1/Hyg/C-FLAG to generate FLAG-tagged PECAM1−ICAM1 hybrid molecules.

PECAM-1 Ig domain deletion mutants and mutants defective in homophilic binding were generated using the following strategy. For each construct, the first fragment was generated using primer *Hin*dIII-CD31-5′ and the respective reverse primer (R), and the second fragment was generated using the respective forward primer (F) and CD31-3′end-*Age*I-AS as the reverse primer. Two fragments were then sewed together via the overlapping sequences and then amplified by *Hin*dIII-CD31-5′ and CD31-3′end-*Age*I-AS. The PCR products were digested with *Hin*dIII and *Age*I and were ligated into *Hin*dIII- and *Age*I-digested pcDNA3.1/Hyg/C-FLAG.

For FLAG-tagged PECAM-1 ΔIg1: CD31-del-Ig1-F, 5′-CATGAAGAGCGGAGTGCCCAGTCCCAGGGT-3′; and CD31-del-Ig1-R, 5′-TGGGCACTCCGCTCTTCATGTCAA-CACTGT-3′.

For FLAG-tagged PECAM-1 ΔIg2: CD31-del-Ig2-F, 5′-CCAAGGTGGGACCAAGAGTGAACTGGTCAC-3′; and CD31-del-Ig2-R, 5′-CACTCTTGGTCCCACCTTGGATG-GCCTCTT-3′.

For FLAG-tagged PECAM-1 ΔIg3: CD31-del-Ig3-F, 5′-GTTCCACATCGAACTATTTTCCAAGCCCGA-3′; and CD31-del-Ig3-R, 5′-AAAATAGTTCGATGTGGAACTTG-GGTGTAG-3′.

For FLAG-tagged PECAM-1 ΔIg4: CD31-del-Ig4-F, 5′-CGAACTGGAAGTATGTGAAATGCTCTCCCA-3′; and CD31-del-Ig4-R, 5′-TTTCACATACTTCCAGTTCGGGCT-TGGAAA-3′.

For FLAG-tagged PECAM-1 ΔIg5: CD31-del-Ig5-F, 5′-GTTTGAGGTCCACGCCAAAATGTTAAGTGA-3′; and CD31-del-Ig5-R,5′-TTTTGGCGTGGACCTCAAACTGGGCAT-CAT-3′.

For FLAG-tagged PECAM-1 ΔIg6: CD31-del-Ig6-F, 5′-GATTTCTATCCTTGCCCCATGGAAGAAAGG-3′; and CD31-del-Ig6-R, 5′-ATGGGGCAAGGATAGAAATCTG-GACCTCAT-3′.

Two PECAM-1 mutants defective in homophilic binding were made. For FLAG-tagged PECAM-1 K116A, the following primers were used: CD31-K116A-F, 5′-GTGAA-CAACGCAGAGAAAACCACTGCAGAG-3′; and CD31-K116A-R, 5′-GGTTTTCTCTGCGTTGTTCACAATCA-CAGT-3′.

For the construction of the PECAM-1 mutant with both Ig domains 2 and 3 deleted, the following primers were used: CD31-del-Ig2,3-F, 5′-CCAAGGTGGGGAACTATTTTC-CAAGCCCGA-3′; and CD31-del-Ig2,3-R, 5′-AAAATAGT-TCCCCACCTTGGATGGCCTCTT-3′.

To generate a hybrid molecule with PECAM-1 Ig domains 2 and 3 replaced with the first and second Ig domains of ICAM-1 (designated as PIG1,2), the following four primers were used in two sewing PCRs. First, PCRs were carried out using primers CD31-1 and CD31-I-Ig1,2-R-A and CD31-I-Ig1,2-F-A and CD31-I-Ig1,2-R-B. After sewing, the product was further sewed with the PCR product obtained using primers CD31-I-Ig1,2-F-B and CD31-2AS: CD31-I-Ig1,2-F-A, 5′-CCAAGGTGGGCCCTCAAAAGTCATCCTGCC-3′; CD31-I-Ig1,2-F-B, 5′-CTACCAGCTCGAACTATTTTC-CAAGCCCGA-3′; CD31-I-Ig1,2-R-A, 5′-CTTTTGAGGG-CCCACCTTGGATGGCCTCTT-3′; CD31-I-Ig1,2-R-B, 5′-AAAATAGTTCGAGCTGGTAGGGGGCCGAGG-3′.

Full-length cDNA of BKRB2 (in pcDNA3.1) was obtained from the UMR cDNA Resource Center (University of Missouri, Rolla, MO). A FLAG-tagged version of BKRB2 was generated by PCR using primers: *Eco*RI-BKRB2-5′-F, 5′-G*GAATTC*ACCATGTTCTCTCCCTGGAAGAT-3′; *Age*I-BKR2B-3′end-AS, 5′-CCG*ACCGGT*CTGTCTGCTCCCT-GCCCA-3′.

Transfection of HEK293 Cells. HEK293 cells were seeded in six-well plates the day before transfection at a density of 8×10^5 cells/well. Transfection was conducted using Effectene transfection reagent (Qiagen). Cells were harvested and lysed for experiments 40 h after transfection.

Cellular Lysate Preparation. Cells were harvested by being scraped in ice-cold PBS and pelleted by centrifugation and were lysed in lysis buffer [50 mM Tris-HCl (pH 7.5), 125 mM NaCl, 60 mM octyl glucoside, and 2 mM sodium vanadate] containing protease inhibitors (Complete Protease Inhibitor tablet, Roche) on ice for 30 min. Lysates were then centrifuged at 14000*g* for 20 min at 4 °C to remove insoluble material. The cleared supernatant was used for experiments.

Transfection of HUVEC with siRNA. HUVEC (7×10^5) were seeded in 35 mm plates the day before transfection. Control siRNA and PECAM-1 siRNA were transfected into cells with X-treme siRNA transfection reagent (Roche) according to the manufacturer's instructions. Transfection mixtures were replaced with 2 mL of culture medium. Cells were starved with M199 medium with 2% bovine serum albumin (BSA) 4 h before the experiments.

Immunoprecipitation. For anti-FLAG immunoprecipitation, transfected cell lysates were incubated with immobilized anti-FLAG M2 monoclonal antibody agarose (Sigma) and rotated at 4 °C for 2 h. For anti-PECAM-1 immunoprecipitation, lysates were first precleared with Protein A beads (Pierce) for 1 h. Six microliters of anti-PECAM-1 rabbit antiserum was added to the lysates, and the samples were rotated at 4 °C overnight. Lysates with preimmune serum added were used as a control. Fifty microliters of Protein A beads was added and the mixture rotated at 4 °C for an additional 2 h. To remove nonspecific bound proteins, the beads were washed four times with lysis buffer. The bound proteins were eluted with 0.1 M glycine buffer (pH 2.5) containing 60 mM octyl glucoside and immediately neutralized with $^1/_{10}$ volume of 1 M Tris buffer (pH 8.0). The proper amount of 4× LDS NuPAGE sample buffer (Invitrogen) and reducing agent was added to each sample. Samples were heated at 95 °C for 10 min before being loaded onto gels.

Immunoblot. Proteins were separated on 4 to 12% NuPAGE gels (Invitrogen) in MOPS running buffer and transferred to PVDF membranes. Following blocking with 5% BSA in Tris-buffered saline (TBS), membranes were incubated with primary antibodies prepared in TTBS/BSA (TBS with 0.1%

Tween 20 and 5% BSA) at room temperature for 1 h. After three 10 min washes with TTBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. For immunoprecipitation experiments, TrueBlot reagent (eBioscience, San Diego, CA) was used to reduce the amount of potential interference from the primary antibody. After being washed, the membranes were incubated in chemiluminescent HRP substrates (SuperSignal West Pico or West Femto substrates from Pierce) and were exposed to X-ray films. Band intensity (mean optical density integrated for the band area) was quantified on unsaturated X-ray films scanned by a digital image analyzer (Quantity-One, Bio-Rad) and quantified with ImageJ. All comparisons were made relative to controls.

Aluminum Fluoride Treatment of Transfected HEK293 Cells. One day after transfection, HEK293 cells were starved overnight with culture medium containing 2% BSA (without FBS). Activation of G protein was achieved by treating cells with starvation medium containing 10 mM NaF and 30 μM $AlCl_3$. Cells added with same amount of starvation medium served as a control. Cells were incubated at 37 °C for 20 min. Medium was removed after incubation, and ice-cold PBS was added to the cells. Cells were harvested and lysates prepared for anit-FLAG antibody immunoprecipitation and Western blot analysis.

Bradykinin Activation of Transfected HEK293 Cells. One day after transfection, HEK293 cells were starved overnight with culture medium (without FBS) containing 2% BSA overnight. Bradykinin prepared in the same medium was added to each well to a final concentration of 100 nM. Cells added with same amount of medium without bradykinin served as a control. Cells were incubated at 37 °C for 5 min. Medium was removed after incubation, and ice-cold PBS was added to the cells. Cells were harvested and lysates prepared for Western blot analysis.

Bradykinin Activation and Hyperosmotic Shock of siRNA-Transfected HUVEC. Three days after siRNA transfection, cells were starved with M199 medium containing 2% BSA for 4 h. Bradykinin prepared in the same medium was added to each well at the desired concentration. For hyperosmotic shock, a 3 M sucrose solution (in starvation medium) was added to each well to produce a final concentration of 300 mM. Cells were incubated at 37 °C for 5 min. Medium was removed after incubation, and ice-cold PBS was added to the cells. Cells were harvested and lysates prepared for Western blot analysis.

RESULTS

PECAM-1 Forms a Complex with Gαq/11. Previously, by means of immunohistochemistry, a PECAM-1-dependent localization of Gαq/11 at the cell−cell junction in the mouse aorta was observed (manuscript submitted). To investigate whether PECAM-1 forms a complex with Gαq/11, immunoprecipitation of PECAM-1 from lysates of freshly harvested HUVEC was performed. Gαq/11 was detected in the rabbit anti-PECAM-1 antiserum immunoprecipitated proteins, but not in the control experiment where preimmune serum was used (Figure 1A). Similar results were observed when immunoprecipitation was performed on lysates of bovine aortic endothelial cells (data not shown). This result

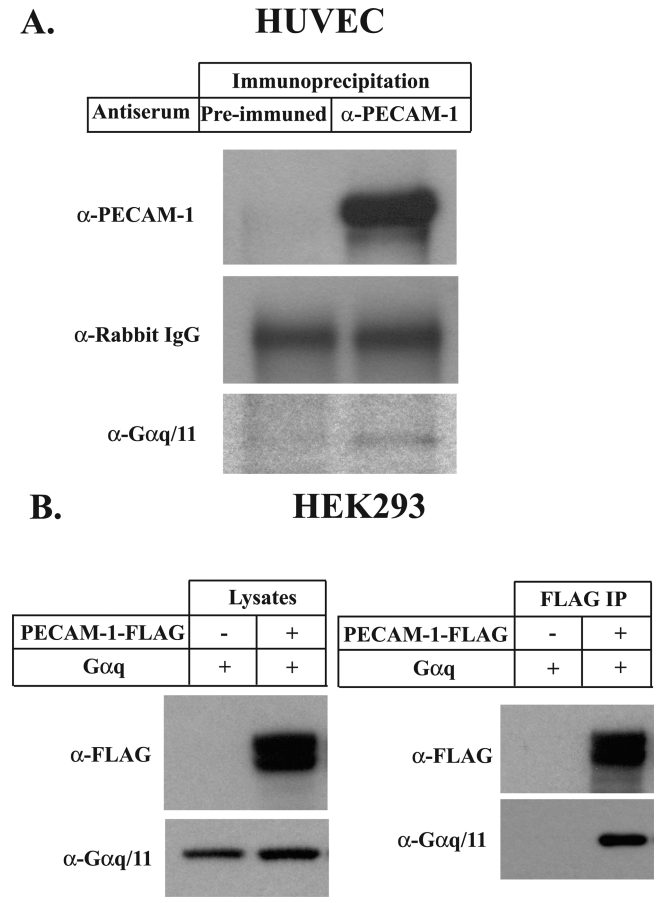

FIGURE 1: PECAM-1 and Gαq form a complex in primary endothelial cells and transfected HEK293 cells. (A) PECAM-1 was immunoprecipitated from HUVEC using anti-PECAM-1 rabbit antiserum. Lysates with preimmune serum added were used as a control. (B) HEK293 cells were transfected with expression vectors containing FLAG-tagged PECAM-1 and Gαq cDNAs. Cells transfected with Gαq cDNA alone were used as a control. Cells were harvested 40 h after transfection. Cell lysates were prepared and immunoprecipitated with anti-FLAG antibody-coupled agarose. Lysates and precipitated proteins were subjected to immunoblot analysis with the indicated antibodies. Bound primary antibodies were detected with corresponding HRP-conjugated secondary antibodies. The membranes were then incubated with HRP chemiluminescent substrate and exposed to X-ray films.

indicated that PECAM-1 and Gαq/11 form a complex in endothelial cells.

To dissect the PECAM-1−Gαq/11 interaction at the molecular level, we investigated such interaction in a heterologus expression system. HEK293 cells were transiently transfected with plasmids encoding FLAG-tagged PECAM-1 and Gαq. As shown in Figure 1B, Gαq was detected in anti-FLAG antibody immunoprecipitates when HEK293 cells were cotransfected with vectors encoding both FLAG-tagged PECAM-1 and Gαq, but not in cells transfected with the Gαq vector alone. These results suggest that PECAM-1 is capable of forming a complex with Gαq.

The Intracellular Domain of PECAM-1 Is Not Required for Formation of the Complex with Gαq. To identify the Gαq interacting region, a series of PECAM-1 cytoplasmic domain deletion mutants were generated. The PECAM-1 cytoplasmic domain is encoded by eight short exons, and its exon organization is highly conserved among species, indicating functional significance (*8, 24*). Therefore, the cytoplasmic domain based on the exon structure was

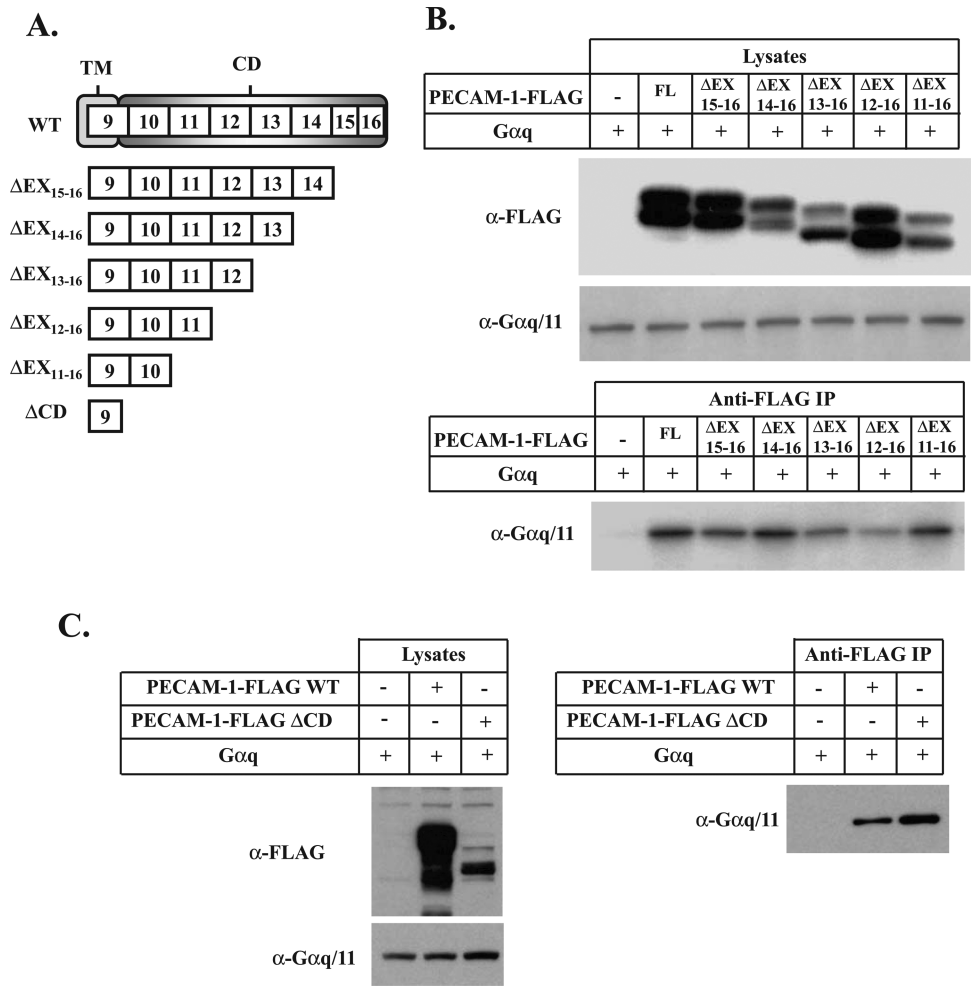

FIGURE 2: PECAM-1 cytoplasmic domain is not required for PECAM-1−Gαq interaction. Panel A shows a schematic presentation of PECAM-1 cytoplasmic domain deletion mutants. Mutants were constructed by sequentially deleting specific exon-encoding regions (indicated by numbers). TM and CD denote sequences encoding the transmembrane and cytoplasmic domains, respectively. (B) HEK293 cells were transfected with expression plasmids encoding Gαq and FLAG-tagged full-length PECAM-1 or one of the cytoplasmic domain deletion mutants as indicated. Forty hours after transfection, cells were harvested, lysed, and subjected to immunoprecipitation with the anti-FLAG antibody. Lysates and immunoprecipitated proteins were subjected to SDS−PAGE. Immunoblot analysis was carried out with the indicated antibodies. (C) HEK293 cells were transfected with expression plasmids encoding FLAG-tagged full-length (WT) and cytoplasmic domain-deleted (ΔCD) PECAM-1. Forty hours after transfection, cell lysates were prepared and subjected to immunoprecipitation with anti-FLAG beads. Immunoblot analysis was carried out with the indicated antibodies. These experiments were performed three times with similar results. Representative blots are shown.

truncated (Figure 2A). Surprisingly, Gαq association was still detected even in mutants from which exon 11 had been deleted (Figure 2B). We further determined the possibility of the remaining region of cytoplasmic domain (10 amino acids of exon 10 and 9 amino acids at the cytoplasmic domain of exon 9) mediating formation of the complex with Gαq. As shown in Figure 2C, deletion of these amino acids (PECAM-1 ΔCD) did not abolish the interaction. This result suggested that the cytoplasmic domain of PECAM-1 is not necessary for its interaction with Gαq.

The possibility of PECAM-1 mediating formation of the complex with Gαq through its extracellular or transmembrane domains was considered next. The extracellular domain of PECAM-1 has been shown to mediate homophilic binding of PECAM-1 from adjacent cells, and the regions critical for this interaction have been mapped to the first and second Ig domains (*3, 5, 25*). Heterophilic binding of PECAM-1 with other proteins through its extracellular domains has also been demonstrated (*26–28*). To test whether the extracellular domain is responsible for formation of the complex with Gαq, domain swapping experiments were performed by swapping PECAM-1 domains with another Ig-like adhesion molecule, ICAM-1, which does not form a complex with Gαq (Figure 3B). To dissect which domain of PECAM-1 mediates the interaction, hybrid molecules were created by swapping the extracellular, transmembrane, and cytoplasmic domains between PECAM-1 and ICAM-1 (Figure 3A). As shown in Figure 3B, constructs which contain the cytoplasmic domain of PECAM-1 did not support its formation of a complex with Gαq. This result was consistent with the results obtained from the cytoplasmic domain deletion mutant experiments (Figure 2). The constructs which contain the extracellular domain of PECAM-1, however, were sufficient to pull down Gαq when they were expressed in HEK293 cells, indicating that the extracellular domain of PECAM-1, but neither the transmembrane nor the intracellular domain, mediates formation of its complex with Gαq.

The PECAM-1 Extracellular Domain Mediates Formation of the Complex with Gαq and Bradykinin Receptor B2. To further investigate whether the extracellular domain of

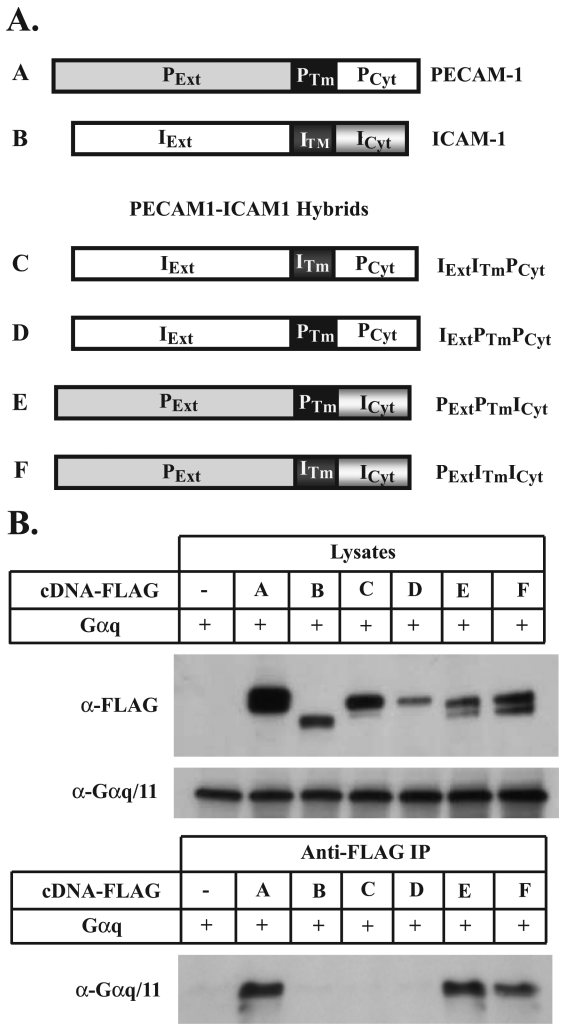

FIGURE 3: Interaction between PECAM-1 and Gαq is mediated by the PECAM-1 extracellular domain. Panel A shows a schematic presentation of domain structures of PECAM-1 and ICAM-1 and hybrid proteins created by swapping domains between PECAM-1 and ICAM-1. P represents PECAM-1, and I represents ICAM-1. The domains are indicated in subscript letters. Ext represents the extracellular domain, Tm the transmembrane domain, and Cyt the cytoplasmic domain. (B) HEK293 cells were transfected with plasmids encoding FLAG-tagged PECAM-1 (A), ICAM-1 (B) and PECAM-1−ICAM-1 hybrid proteins [(C) $I_{Ext}I_{Tm}P_{Cyt}$, (D) $I_{Ext}P_{Tm}P_{Cyt}$, (E) $P_{Ext}P_{Tm}P_{Cyt}$, and (F) $P_{Ext}I_{Tm}P_{Cyt}$]. Forty hours after transfection, cell lysates were immunoprecipitated with the anti-FLAG antibody. Association of Gαq with FLAG-tagged proteins was analyzed by immunoblotting using the anti-Gαq/11 antibody. These experiments were performed three times with similar results. Representative blots are shown.

PECAM-1 is indeed important for formation of the complex with Gαq, we generated a series of Ig domain deletion mutants and examined their ability to form a complex with Gαq when expressed in HEK293 cells. As shown in Figure 4A, all Ig domain deletion mutants maintain their ability to form complexes with Gαq, although a decrease in the amount of Gαq pulled down by Ig domain 2 and 3 deletion mutants was consistently observed. To test the possibility of both Ig domains 2 and 3 contributing to the interaction, two additional mutant constructs were generated. First, a PECAM-1 molecule which lacks both the second and third Ig domains (ΔIg2,3) was generated. In the second construct, the second and third Ig domains of PECAM-1 were replaced

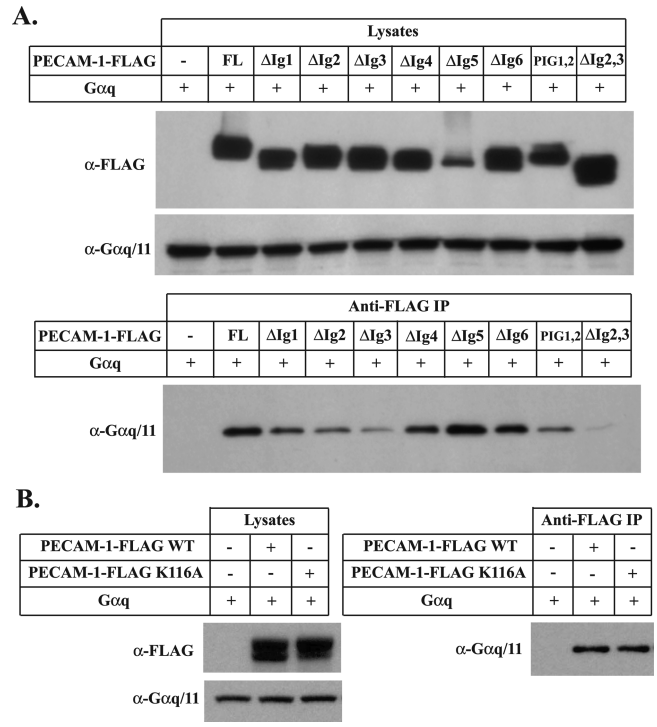

FIGURE 4: Second and third Ig-like domains of PECAM-1 are critical for its interaction with Gαq. (A) HEK293 cells were cotransfected with expression plasmids encoding Gαq and FLAG-tagged full-length (FL) PECAM-1 and its Ig domain deletion mutants. Mutants with a single Ig domain deleted and Ig domains 2 and 3 deleted (ΔIg2,3) were tested. The vector encoding a hybrid protein with the second and third PECAM-1 Ig domains replaced with the first and second ICAM-1 Ig domains, respectively (designated PIG1,2), was also tested. Forty hours after transfection, cells were harvested, lysed, and immunoprecipitated with anti-FLAG-coupled agarose. Immunoblot analysis was carried out to verify the protein expression in lysates, and Gαq association was analyzed on immunoprecipitated proteins. (B) HEK293 cells were cotransfected with expression vectors encoding Gαq and FLAG-tagged wild-type PECAM-1 (WT) or a homophilic binding-deficient mutant, K116A. Formation of the complex of FLAG-tagged proteins with Gαq was assessed as mentioned above. These experiments were performed three times with similar results. Representative blots are shown.

with the first and second Ig domains of ICAM-1, respectively (designated PIG1,2). When it was expressed in HEK293 cells, a further decrease in the level of formation of the Gαq complex was observed in ΔIg2,3 compared to that with Ig domain 2 or 3 deleted (Figure 4A), indicating that both domains contribute to formation of the complex with Gαq. It is noteworthy that deletion of both Ig domains 2 and 3 does not completely abolish formation of the complex between PECAM-1 and Gαq, and replacement of these domains with ICAM-1 Ig domains 1 and 2 (PIG1,2) slightly compensates for the loss (Figure 4A). This suggests a possible contribution from another domain, possibly the first Ig domain, in mediating this interaction. To assess whether homophilic interaction of PECAM-1 is critical for this interaction, a K116A mutant which is defective in its ability to mediate homophilic binding (*5*) was generated. Homophilic binding of PECAM-1 is apparently not critical for the interaction with Gαq for a substantial amount of Gαq was still pulled down by the PECAM-1 K116A mutant (Figure 4B).

Since Gαq is an intracellular protein, the observation that the extracellular domain of PECAM-1 mediates formation

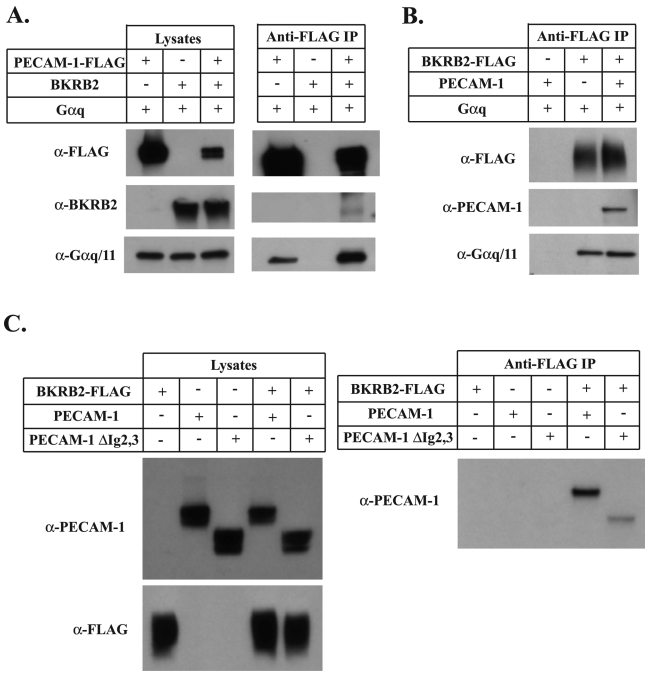

FIGURE 5: Bradykinin receptor B2 forms a complex with PECAM-1 and Gαq. (A) HEK293 cells were transfected with plasmids for FLAG-tagged PECAM-1 and nontagged BKRB2. Lysates and anti-FLAG immunoprecipitated proteins were analyzed by probing with the indicated antibodies. (B) HEK293 cells were cotransfected with plasmids encoding FLAG-tagged BKRB2 and a nontagged PECAM-1. Gαq cDNA was also transfected to assess the association. Anti-FLAG immunoprecipitated proteins were analyzed by immunoblotting with the indicated antibodies. (C) Plasmid encoding FLAG-tagged BKRB2 that was cotransfected with either full-length PECAM-1 (nontagged) or PECAM-1 lacking Ig domains 2 and 3 (ΔIg2,3) (nontagged). Lysates and immunoprecipitated proteins with anti-FLAG agarose were probed with the indicated antibodies.

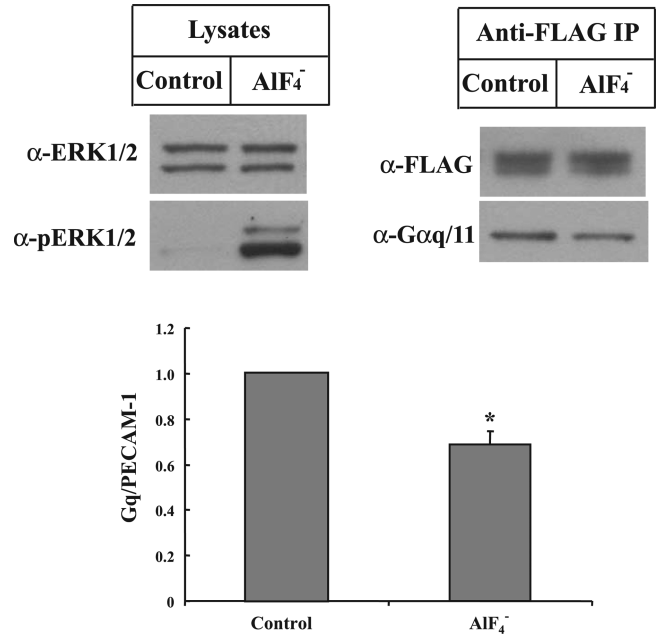

FIGURE 6: Effect of Gαq activation on formation of its complex with PECAM-1. HEK293 cells were transfected with plasmids encoding FLAG-tagged PECAM-1 and nontagged BKRB2. Cells were starved overnight and treated with starvation medium containing 10 mM NaF and 30 μM $AlCl_3$ for 20 min. Lysates and anti-FLAG immunoprecipitated proteins were analyzed by probing with the indicated antibodies. Data are presented as means ± the standard error of the mean, and statistical comparisons between control and AlF_4^--treated groups were performed using the Student's *t* test with a $P < 0.05$ significance level.

of the complex with Gαq indicates that another protein bridges this interaction. Although alternative hypotheses have been suggested in recent years, heterotrimeric G proteins are traditionally thought to associate with GPCRs in their inactive state. Therefore, the hypothesis that a GPCR bridges the interaction between PECAM-1 and Gαq was tested. Bradykinin receptor B2 (BKRB2), a Gαq-coupled GPCR, has been shown to interact with eNOS (*29*), a molecule that has been shown to interact with PECAM-1 (*18, 30*). Furthermore, BKRB2 senses fluid shear stress in endothelial cells (*31*). Therefore, whether BKRB2 is the bridging molecule for formation of the PECAM-1−Gαq complex was tested. As shown in Figure 5A, there was a significant increase in the amount of Gαq immunoprecipitated by anti-FLAG immunoprecipitation in cells expressing both PECAM-1 and BKRB2 compared to that in cells expressing PECAM-1 alone. Anti-BKRB2 antibody reactivity detected in anti-FLAG immunoprecipitated proteins also suggested that these two molecules form a complex (Figure 5A). To confirm this result, a FLAG-tagged BKRB2 construct was coexpressed with a nontagged PECAM-1 construct in HEK293 cells. Immunoprecipitation of BKRB2 with anti-FLAG antibody was able to pull down Gαq (Figure 5B). PECAM-1 was detected when cells were cotransfected with a PECAM-1 expression vector. This result suggested that BKRB2 forms a complex with PECAM-1 and is capable of bridging formation of the complex between PECAM-1 and Gαq in HEK293 cells.

To address the question of whether PECAM-1 Ig domains 2 and 3, which are critical for formation of a complex with Gαq, are also important for the interaction with BKRB2, we tested the interaction of PECAM-1 ΔIg2,3 with FLAG-tagged BKRB2 in HEK293 cells. As shown in Figure 5C, immunoprecipitation of FLAG-tagged BKRB2 was able to pull down the full-length nontagged version of PECAM-1. However, the extent of association of the PECAM-1 ΔIg2,3 mutant with FLAG-tagged BKRB2 was drastically reduced. Taken together, these results confirmed a role of Ig domains 2 and 3 of PECAM-1 in mediating formation of the complex with BKRB2 and G protein α subunit q/11.

Interaction of PECAM-1 with Aluminum Fluoride-Activated Gαq. To determine whether the activation state of Gαq affects the PECAM-1−Gαq complex, transfected HEK293 cells were treated with aluminum fluoride (AlF_4^-). AlF_4^- activates G protein α subunits by binding to the GDP-bound form and mimicking the γ-phosphate of GTP in the transition state intermediate of the GTPase reaction and prevents rapid deactivation of the α subunit by GTP hydrolysis (*32*). AlF_4^- treatment of FLAG-tagged PECAM-1-transfected HEK293 cells caused a reduction (approximately 30%) in the level of Gαq detected in anti-FLAG immunoprecipitated fractions (Figure 6), indicating that activation of G protein α subunits causes partial dissociation of Gαq from the PECAM-1 complex.

PECAM-1 Regulates Bradykinin Receptor Activities through Its IgG Domains 2 and 3 in HEK293 Cells. To investigate the role of PECAM-1 in the BKRB2−Gαq complex, we first tested whether PECAM-1 modulates bradykinin receptor activation. As shown in Figure 7, coexpression of PECAM-1

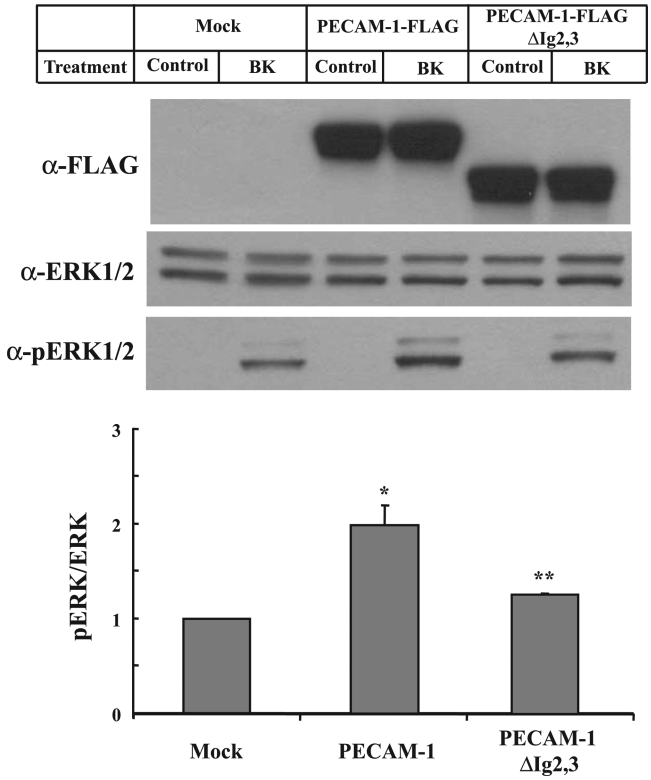

FIGURE 7: PECAM-1 regulates BKRB2 activity through its Ig domains 2 and 3. HEK293 cells were cotransfected with BKRB2 with either full-length PECAM-1 (FLAG-tagged) or PECAM-1 lacking Ig domains 2 and 3 (ΔIg2,3) (FLAG-tagged). Empty vector was cotransfected with BKRB2 cDNA as a control (mock). Thirty hours after transfection, cells were starved overnight. Cells were treated with 100 nM bradykinin (BK) for 5 min and harvested. Cells treated with medium without BK served as a control. Cell lysates were prepared for immunoblot analysis using antibodies against FLAG, ERK1/2, and phospho-ERK1/2. Signals obtained from phospho-ERK1/2 were normalized against that of total ERK1/2, and the values were set to 1 for the control. The experiments were performed three times, and representative blots are shown. Data are presented as means ± the standard error of the mean, and statistical comparisons were performed using the paired *t* test with a $P < 0.05$ significance level (one asterisk for mock-transfected vs PECAM-1-transfected and two asterisks for wild-type PECAM-1-transfected vs PECAM-1 ΔIg2,3-transfected).

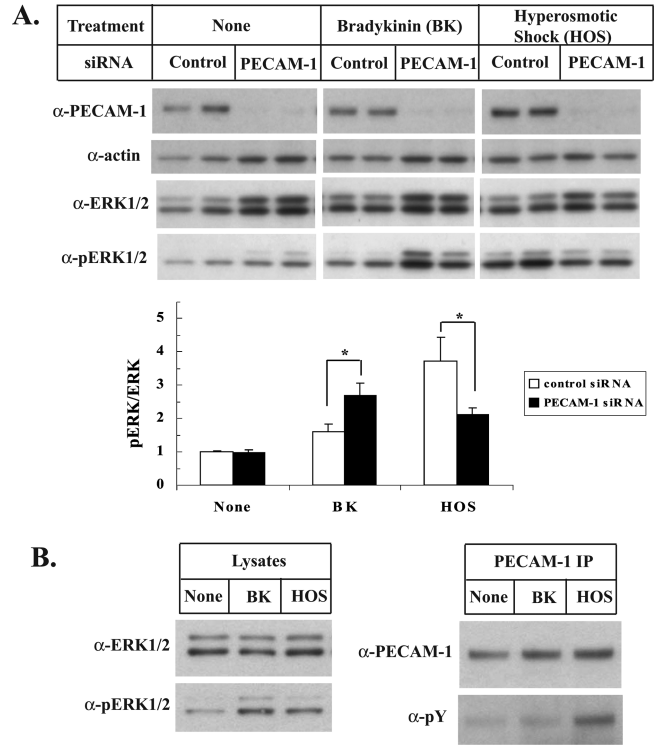

FIGURE 8: PECAM-1 differentially regulates bradykinin- and hyperosmotic shock-induced ERK1/2 activation in HUVEC. (A) HUVEC were transfected with either control siRNA or siRNA against PECAM-1. Three days after transfection, cells were starved for 4 h before the experiments. Cells were treated with either 1 nM bradykinin (BK) or hyperosmotic shock (HOS) for 5 min and harvested. Cells without any treatment (none) served as a control. Cell lysates were prepared for immunoblot analysis using antibodies against PECAM-1, actin, ERK1/2, and phospho-ERK1/2. Signals obtained from phospho-ERK1/2 were normalized against that of total ERK1/2, and the values were set to 1 for the control. The experiments were performed in duplicate. Data are presented as means ± the standard error of the mean, and statistical comparisons between control and PECAM-1 siRNA-treated groups were performed using the Student's *t* test with a $P < 0.05$ significance level. (B) HUVEC treated with BK and HOS were immunoprecipitated with anti-PECAM-1 antibody. Precipitated proteins were probed with anti-PECAM-1 and anti-phosphotyrosine (pY) antibodies. Immunoblot analysis was also carried out on lysates using antibodies against ERK1/2 and phospho-ERK1/2.

with BKRB2 in HEK293 cells enhanced bradykinin-induced extracellular signal-regulated kinase 1/2 (ERK1/2) activation, and this effect was weakened when PECAM-1 ΔIg2,3, a mutant that does not interact well with BKRB2 (Figure 5), was expressed. This result further supports the hypothesis that Ig domains 2 and 3 of PECAM-1 contribute to formation of the complex with BKRB2 and regulate its activity.

PECAM-1 Differentially Regulates Bradykinin- and Hyperosmotic Shock-Induced ERK1/2 Phosphorylation in HUVEC. To investigate the role of PECAM-1 in the BKRB2−Gαq complex, we tested whether PECAM-1 modulates bradykinin receptor activation in endothelial cells. We took the approach of small interference RNA (siRNA)-mediated gene silencing to suppress PECAM-1 expression in HUVEC and examined its effect on bradykinin-induced ERK1/2 activation. As shown in Figure 8A, transfection of HUVEC with siRNA against PECAM-1, but not the control siRNA, reduced the level of PECAM-1 expression by more than 90%. Treatment with 1 nM bradykinin led to an increase in the level of ERK1/2 phosphorylation (Figure 8A,B). In PECAM-1 siRNA-transfected cells, a 50% increase in the level of ERK1/2 phosphorylation compared to that of the cells transfected with control siRNA was observed (Figure 8A). This increase was observed only when a small dose of bradykinin (1 nM) was used (data not shown). To confirm that the augmentation in ERK1/2 phosphorylation in bradykinin-treated PECAM-1 knockout cells was not due to a nonspecific effect from siRNA transfection, another ERK1/2 activation condition known to be PECAM-1-dependent was tested. Osmotic shock has been shown to induce ERK1/2 activation in endothelial cells, and this activation is dependent on the presence of PECAM-1 (*15, 33*). When HUVEC were treated with hyperosmotic shock, an increase in the level of ERK1/2 phosphorylation was observed (Figure 8A,B). However, the degree of phosphorylation was reduced in PECAM-1 siRNA-treated cells compared to that in the control siRNA-treated cells, which is consistent with previously reported results (*15, 33*). Since tyrosine phosphorylation of PECAM-1 is required for hyperosmotic shock-

induced ERK1/2 activation (*15*), we examined whether it is also important for bradykinin-induced ERK1/2 activation. In contrast to hyperosmotic shock treatment, bradykinin treatment did not lead to an increase in the level of tyrosine phosphorylation of PECAM-1 (Figure 8B). Taken together, these results indicated that PECAM-1 regulates bradykinin- and hyperosmotic shock-induced ERK1/2 activation through different mechanisms.

DISCUSSION

Roles of PECAM-1 in signaling through its cytoplasmic domain have been investigated extensively. Many of the PECAM-1-regulated receptors, including T- and B-cell receptors and Fc receptor, contain immunoreceptor tyrosine-based activation motifs (ITAMs). Despite the lack of evidence of direct interaction, PECAM-1 has been demonstrated to negatively regulate the activities of these ITAM-containing receptors using cells derived from PECAM-1 knockout mice (*7*). Since activation of the receptors that were examined leads to tyrosine phosphorylation of PECAM-1, most of these observed effects are attributed to the inhibitory effects of the ITIM domains. Consistent with a previous report by others (*34*), we did not detect an increase in the level of tyrosine phosphorylation upon bradykinin stimulation in HUVEC. Therefore, it is unlikely that the ITIM domains of PECAM-1 play a role in regulating BKRB2 activity that we presented here. The molecular evidence that this interaction is mediated by the extracellular domain of PECAM-1 further supports this notion.

The extracellular domain of PECAM-1, besides mediating the homophilic interaction, has also been shown to mediate heterophilic interactions with proteins, such as integrin $\alpha v\beta 3$, CD38, and CD177 (*27, 35–37*). Here, we demonstrate, using molecular and biochemical approaches, that PECAM-1 forms a complex with BKRB2 through its extracellular domain. Interestingly, the critical region of PECAM-1 for this novel heterophilic interaction, Ig domains 2 and 3, overlaps with the interacting regions previously identified for $\alpha v\beta 3$ and CD38 (Ig domains 1 and 2 for $\alpha v\beta 3$ and Ig domains 1−3 for CD38) (*35, 38*). In transfected HEK293 cells, PECAM-1 is able to regulate BKRB2 activity through this interaction as coexpression of a full-length PECAM-1, but not ΔIg2,3 mutant, with BKRB2 upregulates agonist-induced ERK1/2 activation. In HUVEC, however, PECAM-1 has a negative regulator role in BK activation in HUVEC. This differential response can be attributed to differences in signaling mediators in the two cell types (*39, 40*). For example, two proteins that have been shown to be activated by BK in endothelial cells, eNOS and VEGFR2 (*41–43*), are not present in HEK293 cells. Interestingly, both eNOS and VEGFR2 have also been shown to interact with PECAM-1 (*18, 19, 30*).

A regulatory role of PECAM-1 in regulating GPCR and G protein activity has been suggested previously. For example, a change in Gα13- and Gαi2-mediated cell migration in serum sphingolipid-simulated PECAM-1-deficient endothelial cells has been observed (*44*). A weakened response to thrombin- and histamine-activated Rho in PECAM-1 null endothelial cells compared to that in PECAM-1-reconstituted endothelial cells was also reported (*44*). In PECAM-1 antisense oligonucleotide-treated endothelial cells, there was a pertussis toxin-sensitive induction of tissue factor expression upon thrombin treatment, indicating that PECAM-1 modulates the thrombin-mediated Gαi/o signaling pathway (*45*). Furthermore, compared to an irrelevant isotype-matched antibody, cross-linking with the anti-PECAM-1 antibody inhibited thrombin-induced platelet aggregation, although the effect could be observed only when a small dose of thrombin (0.05 unit/mL) was used (*46*). This result was similar to what we observed in PECAM-1 silencing experiments where the BKRB2 regulatory activity of PECAM-1 can be seen only with a small dose of BK, suggesting that either this interaction is weak or only a small portion of GPCRs is modulated, directly or indirectly, by PECAM-1. It is noteworthy that PECAM-1 and Gαq form a complex in HEK293 cells even without additional expression of BKRB2. It is likely that endogenous Gαq-coupled GPCRs expressed by HEK293 cells bridge the interaction between PECAM-1 and Gαq. At present, we are focusing on investigating whether the ability of PECAM-1 to interact with and regulate BKRB2 can be extended to other GPCRs. In this regard, it is interesting to note that endothelial nitric oxide synthase (eNOS), a protein that we have previously shown to form a complex with PECAM-1 (*18, 30*), has been shown to interact with several GPCRs, including BKRB2, endothelin receptor, and angiotensin receptor, but not with the P2Y2 receptor (*29*). Although we previously demonstrated that the cytoplasmic domain of PECAM-1 was able to inhibit eNOS activity, the concentration of the GST−CD31 cytoplasmic domain fusion protein used to reach 50% inhibition of maximal eNOS activity was 1 order of magnitude higher than that of the GST−GPCR ID4 domain (intracellular domain 4) fusion proteins used in the aforementioned study (25 μM vs 1 μM). This indicates a stronger affinity between eNOS and GPCR ID4 than between eNOS and the PECAM-1 cytoplasmic domain. We are currently investigating whether PECAM-1 interacts with eNOS directly or through the bridging of GPCRs.

It has been shown that homophilic binding of PECAM-1 is crucial for its mechanosensing ability (*15*). Here we have demonstrated that Ig domains 2 and 3 of PECAM-1 are critical for BKRB2 interaction and homophilic engagement of PECAM-1 is not required for its interaction. Although we cannot rule out this possibility at this point, it is conceivable that these domains are only capable of mediating either homophilic or heterophilic interaction at a given time. Although PECAM-1 is predominately localized at the cell−cell junction in the confluent state, it is also present at the EC luminal side, possibly in its monomeric form (*47*). It is possible that the monomeric form of PECAM-1 is responsible for heterologous interaction with other proteins. In fact, by immunostaining, we did not observe a colocalization of BKRB2 and PECAM-1 at cell−cell junctions in HUVEC (data not shown), despite the fact that we have previously found that Gαq localizes at endothelial cell−cell junctions in a PECAM-1-dependent manner (manuscript submitted). It is likely that other additional cell−cell junction-localized GPCRs or other molecules are responsible for mediating formation of the PECAM-1−Gαq complex at endothelial junctions.

Taken together, our data demonstrate a novel interaction between PECAM-1 and the BKRB2−Gαq complex. Our results also indicate that PECAM-1 regulates BKRB2 activity and mechanotransduction in differential manners. The de-

tailed mechanism of how PECAM-1 regulates the BKRB2 activity is now being investigated in our laboratory.

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