A Novel Protein-Protein Interaction between a G Protein-coupled Receptor and the Phosphatase SHP-2 Is Involved in Bradykinin-induced Inhibition of Cell Proliferation*

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Mitogenic G protein-coupled receptor (GPCR) signaling has been extensively studied. In contrast, little is known about anti-mitogenic GPCR signaling. We show here that anti-mitogenic signaling of a GPCR, the bradykinin B2 receptor, involves a novel direct protein-protein interaction. The antiproliferative effect of bradykinin was accompanied by a transient increase in protein-tyrosine phosphatase activity. Using surface plasmon resonance analysis, we observed that an immune receptor tyrosine-based inhibitory motif (ITIM) located in the C-terminal part of the B2 receptor interacted specifically with the protein-tyrosine phosphatase SHP-2. The interaction was confirmed in primary culture renal mesangial cells by co-immunoprecipitation of a B2 receptor-SHP-2 complex. The extent of the interaction was transiently increased by stimulation with bradykinin, which was accompanied by an increase in specific SHP-2 phosphatase activity. Mutational analysis of the key ITIM residue confirmed that the B2 receptor ITIM sequence is required for interaction with SHP-2, SHP-2 activation, and the anti-mitogenic effect of bradykinin. Finally, in mesangial cells transfected with a dominant-negative form of SHP-2, bradykinin lost the ability to inhibit cell proliferation. These observations demonstrate that bradykinin inhibits cell proliferation by a novel mechanism involving a direct protein-protein interaction between a GPCR (the B2 receptor) and SHP-2.

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\‡ The abbreviations used are: GPCRs, G protein-coupled receptors; MAPK, mitogen-activated protein kinase; TKR, tyrosine kinase receptor; PTP, protein-tyrosine phosphatase; ITIM, immunoreceptor tyrosine-based inhibitor motif; SH2, Src homology-2; FCS, fetal calf serum; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; CHO, Chinese hamster ovary.

G protein-coupled receptors (GPCRs)\‡ compose the largest protein family in eukaryotes. Activation of these seven-transmembrane receptors is known to induce a large panel of biological reactions, among which the stimulation of cell proliferation has been extensively studied (1–4). Mitogenic GPCR signaling involves G protein-dependent activation of mitogen-activated protein kinase (MAPK), recruitment of arrestin in the activation Grb2/Sos, or tyrosine kinase receptor (TKR) transactivation by a still largely unknown mechanism (5). In contrast, only recently have anti-mitogenic effects of GPCRs been discovered (6–8). The bradykinin B2 receptor is a GPCR that is either mitogenic or anti-mitogenic, depending on the experimental conditions.

Bradykinin induces proliferation of quiescent cells via B2 receptor activation (9–11). In contrast, under proliferating conditions, bradykinin is antiproliferative in a variety of different cell types, including breast stromal cells, arterial smooth muscle cells, and mesangial cells (12–14). In renal mesangial cells, this antiproliferative effect of bradykinin is associated with both dephosphorylation of tyrosine residue-containing proteins (15) and increased total tyrosine phosphatase activity (14). Furthermore, in A431 cells, bradykinin increases tyrosine phosphatase activity, diminishes tyrosine phosphorylation of the epidermal growth factor receptor, and reduces epidermal growth factor-induced MAPK activity (16). These effects strongly suggest activation of a protein-tyrosine phosphatase (PTP) in anti-mitogenic bradykinin signaling, but the way by which PTP activity is modulated is not known.

We have identified a consensus immunoreceptor tyrosine-based inhibitor motif (ITIM) (17) in the B2 receptor (Fig. 1), which suggests possible PTP activation via a direct protein-protein interaction between the B2 receptor and the PTP. Upon phosphorylation of the key tyrosine residue in the ITIM sequence, ITIM-bearing receptors (first characterized in hematopoietic receptors) (17) recruit the Src homology-2 (SH2) domains of phosphatases via a direct protein-protein interaction. This interaction activates the phosphatase, which consequently modifies phosphorylation-dependent signaling cascades. The B2 receptor ITIM is located at the interface of the C-terminal part of the seventh transmembrane region and the C-terminal intracellular tail of the bradykinin B2 receptor and is 100% conserved in man, mouse, rabbit, rat, and pig (Fig. 1). The PTPs that potentially interact with the B2 receptor ITIM sequence and that modify the cellular tyrosine phosphorylation content are the cytoplasmic PTPs SHP-1 and SHP-2. These PTPs contain two N-terminal SH2 domains capable of interacting with an ITIM sequence and one C-terminal tyrosyl phosphatase domain (18).

SHP-1 is expressed mainly in hematopoietic cells, whereas SHP-2 is ubiquitously expressed (19). SHP-1 is described as a negative regulator of signaling pathways leading to cell proliferation (20, 21), whereas SHP-2 both negatively and positively regulates proliferation signaling pathways (21, 22).
B2 receptor ITIM sequence

Consensus ITIM sequence

Fig. 1. Alignment of the C-terminal sequences of the B2 receptors from different species shows a 100% conserved ITIM sequence. Sequences were aligned using ClustalW software. Conserved residues are marked by asterisks, and the key ITIM residues are in boldface. TM7, putative seventh transmembrane helix (in gray); x, any amino acid.

In this study, using BIAcore technology, mutational analysis, co-immunoprecipitation studies, and a dominant-negative SHP-2 mutant, we show the existence of a novel direct protein-protein interaction between the tyrosine-phosphorylated ITIM sequence of the B2 receptor and the tyrosine phosphatase SHP-2. The presence of a functional B2 receptor ITIM sequence and catalytically active SHP-2 is necessary to mediate the antiproliferative effect of bradykinin.

EXPERIMENTAL PROCEDURES

Materials

Primary culture mesangial cells were obtained and cultivated in Dulbecco's modified Eagle's medium as described (23). Dulbecco's modified Eagle's medium, α-minimal essential medium, Ham's F-12 medium, streptomycin, penicillin, and fetal calf serum (FCS) were from Invitrogen. Bradykinin and chemical products were from Sigma. Anti-dilution, streptomycin, penicillin, and fetal calf serum (FCS) were from Invitrogen. Bradykinin and chemical products were from Sigma. Anti-B2R human 293

B2R mouse 295

B2R rabbit 296

B2R rat 295

B2R pig 293

| B2R human | 293 | MAY S N S C L N P | L V Y V | G K R F R K K S W E V Y Q |
| B2R mouse | 295 | V A Y S N S G L N P | L V Y V | G K R F R K K S R E V Y R |
| B2R rabbit | 296 | M G Y S N S C L N P | L V Y V | G K R F R K K S R E V Y R |
| B2R rat | 295 | V A Y S N S C L N P | L V Y V | G K R F R K K S R E V Y Q |
| B2R pig | 293 | L A Y S N S C L N P | L V Y V | G K R F R K K S R E V Y H |

| Alignment of the C-terminal sequences of the B2 receptors from different species shows a 100% conserved ITIM sequence. Sequences were aligned using ClustalW software. Conserved residues are marked by asterisks, and the key ITIM residues are in boldface. TM7, putative seventh transmembrane helix (in gray); x, any amino acid.

Sequence analyses were performed with the ClustalW program. Conserved residues are marked by asterisks, and the key ITIM residues are in boldface. TM7, putative seventh transmembrane helix (in gray); x, any amino acid.

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Fig. 2. Antiproliferative effect of bradykinin is accompanied by an increase in total PTP activity. Mesangial cells cultivated with 0.5% (A–C) or 15% (D–F) FCS were incubated with 100 nM bradykinin (BK) for the indicated times. A and D, total PTP activity was determined using 33P-labeled poly(Glu,Tyr). Data are expressed as the percentage of free 33P observed in untreated cells (t = 0). Basal total PTP activities in quiescent and proliferating untreated cells were 105 ± 8 and 110 ± 8 cpm/min of 33P released, respectively. Bradykinin treatment increased total PTP activity in proliferating cells to a maximum of 145 ± 13 cpm/min, B and E, total tyrosine phosphoprotein content was determined by Western blotting with an anti-phosphotyrosine antibody. C and F, shown are the effects of bradykinin on cell proliferation. Cells were cultivated in Dulbecco’s modified Eagle’s medium containing 0.5 or 15% FCS and treated for 24 h with 100 nM bradykinin in medium (black bars) or with medium only (white bars). Data are expressed as the percentage of control values obtained with unstimulated cells. Results are expressed as the means ± S.E. of three independent experiments. **, p < 0.05, significantly different from control values (unstimulated cells; Student’s t test). BK, bradykinin.

EDTA, 1 mM EGTA, 2 mM Na2VO4, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 0.36 μg/ml phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor, 0.01% leupeptin, and 0.01% aprotinin). The mixture was gently agitated for 30 min at 4 °C and centrifuged at 15,000 × g for 15 min at 4 °C, followed by collection of the supernatant.

Immunoprecipitation—500 μg of soluble proteins were incubated with 1.5 μg of antibody for 30 min at 4 °C. The mixture was incubated for 2 h with protein A- or protein G-Sepharose beads prewashed with radioimmune precipitation assay buffer. The mixture was then washed three times with radioimmune precipitation assay buffer and resuspended in 32.5 μl of Laemmli buffer for immunoblotting or washed with 500 μl of PTP buffer (50 mM Tris-HCl (pH 7.0), 0.5 mg/ml bacitracin, and 0.1% bovine serum albumin) and resuspended in 400 μl of PTP buffer containing genistein at 10 μM for PTP activity determination.

Immunoblotting—Solubilized or immunoprecipitated proteins in Laemmli buffer (incubated for 6 min at 100 °C) were loaded onto a 10% SDS-polyacrylamide gel; transferred to a nitrocellulose membrane; and immunoblotted with anti-SHP-1 (1:500 dilution), anti-SHP-2 (1:2500), anti-B2 receptor (1:500), anti-phospho-ERK1/2 (1:3000), or anti-total ERK1 (1:1500) antibodies. Immunoreactive proteins were visualized by ECL immunodetection (Amer sham Bioscience) and quantified by densitometric analysis.

PTP Activity Assay—The poly(Glu,Tyr) substrate (Sigma) was phosphorylated with γ32P-ATP as described (31). 2.5 μg of total lysate (in 60 μl of PTP buffer) or SHP-2 immunoprecipitates (200 μl of the 400 μl described above) were incubated with 30,000 cpm of 32P-labeled poly(Glu,Tyr) in the presence of 50 mM dithiothreitol for 10 min at 30 °C. The reaction was stopped by addition of 1 volume of ice-cold 30% trichloroacetic acid and incubated for 30 min on ice. After centrifugation at 13,000 × g for 10 min at 4 °C, 1 volume of ammonium molybdate was added to 1 volume of supernatant, and the mixture was incubated for 10 min at 30 °C. Then, 2 volumes of isobutyl alcohol/ethanol (50:50) were added, and the solution was thoroughly mixed. The amount of inorganic γ32P extracted using this method was counted with a liquid scintillation counter (Packard Instrument Co.).

Cell Proliferation Assay

Cells were seeded in 12-well plates (Falcon) at 10,000 cells/well and cultivated for 48 h with different media containing 0, 10 or 15% of FCS as indicated in the various experiments. The cells were then stimulated with medium containing 100 nM bradykinin or with medium only. Cell growth was measured after 24 h by counting cells with a Coulter counter ZM (Beckman Coulter, Roissy, France).

RESULTS

Bradykinin-induced Growth Inhibition Is Accompanied by an Increase in Total PTP Activity—Depending on the culture conditions, activation of the bradykinin B2 receptor either stimulates (0.5% FCS, quiescent cells) or inhibits (15% FCS, proliferating cells) mesangial cell proliferation (15). Furthermore, inhibition of cell proliferation by bradykinin is accompanied by a decrease in tyrosine phosphorylation of total cellular protein (14). To investigate whether PTP activity could be involved in the bradykinin-induced decrease in total tyrosine phosphorylation, we determined the time course of total PTP activity in bradykinin-stimulated quiescent and proliferating mesangial cells. In quiescent cells, bradykinin had no effect on total tyrosine phosphatase activity (Fig. 2A). However, under these conditions, bradykinin increased global tyrosine phosphoprotein levels (Fig. 2B), whereas it induced cell proliferation by 27 ± 4% (Fig. 2C). In comparison, addition of FCS for 24 h to quiescent mesangial cells increased cell proliferation by 90 ± 6% (data not shown). In contrast, in proliferating cells, bradykinin inhibited cell proliferation (Fig. 2F) and induced a rapid (0.5 min) 25 ± 7.5% increase in PTP activity, followed by a progressive decrease in PTP activity to the basal value at 5 min (Fig. 2D). This increase in PTP activity was accompanied by a decrease in total tyrosine phosphoprotein content (Fig. 2E). These results suggest that the antiproliferative effects of bradykinin could be mediated by the modification of PTP activity.

The Tyrosine-phosphorylated ITIM Sequence of the B2 Receptor Interacts with the PTP SHP-2—The B2 receptor possesses a highly conserved ITIM sequence (LVY307VIV) (Fig. 1) located on the cytoplasmic side of the seventh transmembrane domain region, which suggests a possible interaction with the SH2 domains of a PTP. Therefore, using surface plasmon resonance,
we investigated whether recombinant SHP-1 or SHP-2 can interact with synthetic peptides containing the ITIM sequence of the B2 receptor.

For this, we purified recombinant GST-PTP fusion proteins from E. coli. Protein purity as analyzed by SDS-PAGE and Coomassie staining was ~95% for the different preparations (data not shown). A clear interaction between the immobilized phosphorylated ITIM peptide and SHP-2 was observed, whereas no significant binding was found for SHP-1 (Fig. 3A). The lack of binding of SHP-1 to the B2 receptor ITIM peptide was not caused by the quality of the SHP-1 preparation because SHP-1 bound to a tyrosine-phosphorylated killer cell inhibitory receptor ITIM peptide (Fig. 3D) (31). Neither SHP-2 nor SHP-1 bound to the non-tyrosine-phosphorylated B2 receptor ITIM peptide (Fig. 3B). SHP-2 lacking the phosphatase domain was still able to bind to the phosphorylated ITIM peptide (Fig. 3A, SHP-2 (SH2)) with 10-fold lower affinity compared with the complete SHP-2 enzyme (Table I). Binding of recombinant SHP-2 was dose-dependent (1–200 nM) (Fig. 3C). Thus, the PTP SHP-2 (but not SHP-1) is able to directly bind, via SH2 domains, to the tyrosine-phosphorylated ITIM sequence of the bradykinin B2 receptor.

The Bradykinin B2 Receptor Interacts with SHP-2 in Primary Culture Rat Mesangial Cells—To determine whether the interaction between the B2 receptor ITIM sequence and SHP-2 also exists in a physiologically relevant cell context, we performed immunoprecipitation experiments on primary culture mesangial cell lysates prepared from proliferating cells, followed by Western blot analysis of the immunoprecipitates. The B2 receptor is known to be present in rat mesangial cells (23), and we identified the presence of SHP-2 in this cell type by Western blot analysis (Fig. 4A). Immunoblotting with anti-B2 receptor antibodies of immunoprecipitates from cell lysates using anti-SHP-2 antibodies revealed co-immunoprecipitation of the B2 receptor and SHP-2 (Fig. 4B). In contrast, immunoprecipitation with SHP-1, present in small amounts in mesangial cells compared with SHP-2 (Fig. 4A), did not result in co-immunoprecipitation of the B2 receptor (Fig. 4B). The interaction in mesangial cells between the B2 receptor and SHP-2 was confirmed by immunoprecipitation with anti-B2 receptor antibodies and immunoblotting of the immunoprecipitate with anti-SHP-2 antibodies (Fig. 4B). The specificity of the antibodies was verified with nonimmune serum (data not shown).

These experiments show that SHP-2 (and not SHP-1) is associated with the B2 receptor in non-bradykinin-stimulated proliferating mesangial cells.

Bradykinin Modifies the Interaction between the B2 Receptor and SHP-2, Which Correlates with SHP-2 Activation—To determine whether the basal interaction between the B2 receptor and SHP-2 could be modified by bradykinin treatment, prolifer-
erating mesangial cells were incubated for different periods with the B2 receptor agonist bradykinin. As shown in Fig. 5 (A and C), bradykinin induced a rapid and significant increase in B2 receptor-SHP-2 complex formation 30 s after initiation of bradykinin treatment (240 ± 12% association compared with basal association), followed by dissociation of the complex at 2 min (59 ± 16% association). 10 min after stimulation with bradykinin, the association between the B2 receptor and SHP-2 returned to a value close to the basal B2 receptor/SHP-2 association (87 ± 14%). Stripping and reblotting of the membrane with anti-SHP-2 antibodies showed that the amount of immunoprecipitated SHP-2 was comparable between the samples. The interaction between the B2 receptor and SHP-2 is thus dynamically modified by B2 receptor stimulation.

The increased B2 receptor/SHP-2 binding observed 30 s after bradykinin treatment correlated with a rapid and transient increase in specific SHP-2 activity (Fig. 5E). SHP-2 activity increased 30 s after bradykinin addition to 125 ± 1.5%, followed by a progressive decrease in SHP-2 activity, reaching 72 ± 14% of the basal value at 10 min.

A basal interaction between the B2 receptor and SHP-2 was also observed in quiescent mesangial cells, but was signifi-
Direct Interaction between Bradykinin B2 Receptor and SHP-2

Fig. 6. A, wild-type and Y307F mutant B2 receptor (B2R) mRNA expression analysis in stably transfected CHO cells by reverse transcription-PCR; B and C, mutation of Y307F does not modify B2 receptor activation in quiescent CHO-DG44 cells. Shown are bradykinin-induced prostaglandin E₂ (PGE₂) release (B) and ERK1/2 activation (ratio of phosphorylated ERK1/2 to total ERK1) (C). Cells were serum-starved in α-minimal essential medium for 48 h and treated for 10 min with 100 nM bradykinin in medium (black bars) or with medium only (white bars). Data are expressed as the percentage of control values obtained with unstimulated cells. Results are expressed as the means ± S.E. of three independent experiments. **, p < 0.05, significantly different from control values (unstimulated cells; Student's t test). Mock, cells transfected with expression plasmid only; WT, cells transfected with an expression plasmid containing the wild-type rat B2 receptor; Y307F, CHO cells transfected with an expression plasmid containing the rat B2 receptor carrying mutation Y307F; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

...cantly less important than in proliferating cells (Fig. 5, B, lanes 1 and 3; and D, bars 1 and 3). Moreover, in quiescent mesangial cells, bradykinin induced only a modest recruitment of SHP-2 to the B2 receptor, which was comparable to the level of the basal interaction between the B2 receptor and SHP-2 in proliferating mesangial cells (Fig. 5, B, lanes 2 and 3; and D, bars 2 and 3). In contrast to proliferating mesangial cells, this reduced bradykinin-induced SHP-2 recruitment to the B2 receptor in quiescent mesangial cells did not result in specific SHP-2 activation (data not shown). Specific SHP-2 activity was inhibited (by 55 ± 8% at 10 min) by the tyrosine phosphatase inhibitor orthovanadate (Fig. 5F). Taken together, these results suggest that bradykinin-induced recruitment of SHP-2 by the B2 receptor activates SHP-2 phosphatase activity in proliferating mesangial cells.

A Functional B2 Receptor ITIM Sequence Is Required for Interaction with SHP-2, SHP-2 Activation, and Antiproliferative Bradykinin Signaling—Mutational analysis was performed to confirm the existence of a direct interaction between the bradykinin B2 receptor and SHP-2. CHO-DG44 cells expressing no functional B2 receptors and transfected with different rat B2 receptor constructs (mock-transfected, wild-type, and ITIM-mutated (Y307F)) were used. Reverse transcription-PCR analysis (Fig. 6A) and bradykinin-induced prostaglandin E₂ production (Fig. 6B) and MAPK activation (Fig. 6C) of the different clones showed that the Y307F mutation did not change the expression or the functionality of the mutant receptor. Moreover, bradykinin induced proliferation only in quiescent wild-type and Y307F cells (data not shown). Furthermore, these experiments confirmed the absence of the B2 receptor in mock-transfected CHO-DG44 cells. Western blot analysis confirmed the overexpression of the B2 receptor (data not shown). These different clones are thus suitable tools to study the role of the ITIM sequence in the interaction between the B2 receptor and SHP-2.

Using immunoprecipitation, we observed that mutation of the key ITIM residue (Y307F) abolished the interaction between the B2 receptor and SHP-2 (Fig. 7A) and bradykinin-induced SHP-2 activation (Fig. 7C). As observed in primary culture mesangial cells, a clear basal interaction between the wild-type B2 receptor and SHP-2 was detected in transfected CHO-DG44 cells, which was transiently increased by bradykinin treatment (Fig. 7B), although this activation was slightly delayed compared with that in mesangial cells. This was accompanied by a transient increase in specific SHP-2 activity (Fig. 7D).

CHO-DG44 cells stably transfected with the B2 receptor were used to determine the contribution of the interaction between the bradykinin B2 receptor and SHP-2 to the antiproliferative effect of bradykinin. The three different clones were cultivated in the presence of FCS (10%) and treated with and without bradykinin (100 nM) for 24 h, followed by cell number determination. Bradykinin treatment inhibited cell proliferation by 33 ± 6% in wild-type cells, whereas it was without effect in mock-transfected and Y307F cells (Fig. 7E). Because the B2 receptor ITIM sequence is highly conserved among species, we investigated whether mutation of the ITIM sequence of the human B2 receptor results in the same phenotype. In a CHO-K1 cell line expressing the human wild-type B2 receptor, a 24 ± 4% inhibition of cell proliferation was observed after bradykinin treatment, whereas this effect was absent in cells expressing the human mutant B2 receptor (Fig. 7F). Human B2 receptor expression as determined by ³H]bradykinin binding was comparable between the human wild-type and Y305A mutant clones and close to the values observed in primary culture mesangial cells (data not shown). These experiments demonstrate the critical role of the rat and human B2 receptor ITIM sequences in the interaction between the receptor and SHP-2, SHP-2 activation, and transduction of antiproliferative bradykinin signals.

A Dominant-negative Mutant of SHP-2 Inhibits Antiproliferative Bradykinin Signaling—Finally, to confirm the importance of SHP-2 in the antiproliferative effect of bradykinin, we used mesangial cells (which express the B2 receptor and SHP-2) stably transfected with different SHP-2 constructs: mock-transfected, wild-type, and a dominant-negative mutant in which the active-site cysteine at position 459 was mutated to serine, resulting in a catalytically inactive PTP (C459S) (30). The three different clones were cultivated in the presence of FCS (10%) and treated with and without bradykinin (100 nM) for 24 h, followed by cell number determination. As previously described for untransfected mesangial cells (15), bradykinin inhibited cell proliferation in mock-transfected mesangial cells by 36%. Interestingly, the inhibitory effect of bradykinin was even larger (48%) in mesangial cells overexpressing wild-type SHP-2. In contrast, bradykinin was without effect on proliferation of mesangial cells.
transfected with the dominant-negative mutant form of SHP-2 (Fig. 8A). In these cells, bradykinin still increased the interaction between the B2 receptor and SHP-2, but the interaction was not transient as with wild-type SHP-2 and was sustained for at least 10 min (Fig. 8B). As expected in these cells, bradykinin was without effect on specific SHP-2 activity (Fig. 8C). These results confirm involvement of SHP-2 in the antiproliferative effect of bradykinin.

DISCUSSION

Activation of the bradykinin B2 receptor, which belongs to the GPCR family, inhibits cell proliferation. This inhibitory effect is associated with an increase in tyrosine phosphatase activity and a decrease in tyrosine phosphorylation (14, 15). In this study, we show for the first time a direct protein-protein interaction between a GPCR and the tyrosine phosphatase SHP-2. This interaction is directly involved in the anti-mitogenic effects of bradykinin.

A highly conserved ITIM sequence located at the interface of the C-terminal part of the seventh transmembrane region and the C-terminal intracellular tail of the B2 receptor was identified. Upon tyrosine phosphorylation, this type of sequence potentially recruits SH2 domain-containing phosphatases. Using
surface plasmon resonance, we found that a phosphorylated B2 receptor ITIM peptide bound with nanomolar affinity (comparable to the affinity of SHP-2 for the ITIM peptide of platelet endothelial cell adhesion molecule-1) (32) to the tyrosine phosphatase SHP-2, but not to SHP-1. This interaction was confirmed in primary culture mesangial cells and in CHO cells by mutational analysis of the key ITIM residue Tyr307. This selective affinity for one of the two tyrosine phosphatases suggests that the amino acid sequence adjacent to the ITIM may be important for selective binding of SHP-2 SH2 domains. Selective tyrosine phosphatase binding (i.e. SHP-1 or SHP-2) was also observed for other ITIM-bearing receptors, including the mutant killer cell inhibitory receptor (33) and Fcγ receptor IIb (34).

The B2 receptor and SHP-2 were found to interact in unstimulated primary culture mesangial cells. This basal interaction suggests ITIM tyrosine phosphorylation of part of the B2 receptor population in the absence of B2 receptor agonist and might correspond to the low spontaneous receptor activity (measured by phosphoinositide hydrolysis) observed for the human B2 receptor in HEK293 cells (35). This basal interaction between the B2 receptor and SHP-2 was transiently increased after stimulation by bradykinin and correlated with a rapid and transient increase in specific SHP-2 phosphatase activity. The kinetics and amplitude of total PTP activity and specific SHP-2 activity were very similar, suggesting that SHP-2 represents the unique PTP activated by bradykinin B2 receptor stimulation under proliferating conditions. The phosphatase activity of SHP-2 in the absence of a tyrosine-phosphorylated binding partner is inhibited by binding of its N-terminal SH2 domain to the phosphatase domain. It has been shown that, upon ITIM receptor tyrosine phosphorylation, the N-terminal SH2 domain changes partners and activates the SHP-2 activity of the enzyme (18, 36). The close correlation between SHP-2 binding and phosphatase activation suggests that binding of SHP-2 to the B2 receptor disrupts the intramolecular interaction and activates the enzyme.

The current data are the first example of direct recruitment and subsequent activation of a tyrosine phosphatase by a GPCR. Such a direct protein-protein interaction of an intracellular protein (other than G proteins) with GPCRs has been observed for endothelial nitric-oxide synthase with the B2, AT1, and endothelin receptors (37); for neuronal nitric-oxide synthase with the B2 receptor (38); for c-Src with the β2-adrenergic receptor (39); and for phospholipase Cγ1 with the AT1 and B2 receptors (40, 41). Although a direct interaction between an intracellular protein and a GPCR was found in all of these studies, only two of them identified the actual sequence motif involved in this interaction: a YIPP motif in the C-terminal domain of the AT1 receptor for phospholipase Cγ1 (40) and proline-rich motifs (PXXP) in the third intracellular loop and C terminus of the β2-adrenergic receptor for c-Src (39). We propose here the involvement of an ITIM sequence in the direct interaction between a GPCR and the tyrosine phosphatase SHP-2.

Mutation of the key tyrosine residue of the B2 receptor ITIM sequence abolished the interaction between SHP-2 and the B2 receptor and the antiproliferative effect of bradykinin. How does B2 receptor-induced SHP-2 activation inhibit TKR-induced cell proliferation? SHP-2 has been shown to both negatively and positively modify TKR-activated signaling pathways. SHP-2 negatively regulates insulin-like growth factor-1 signaling by direct dephosphorylation of the activated insulin receptor in the presence of SHPS-1 (42) or dephosphorylation of downstream signaling molecules such as insulin receptor substrate-1 (43). Furthermore, it has been shown that SHP-2 is involved in growth hormone receptor, JAK2, and STAT5B (signal transducer and activator of transcription) dephosphorylation and inhibition of JAK2 activation (44). Therefore, SHP-2 seems to negatively regulate TKR signaling when bound to phosphorylated TKRs or phosphorylated associated proteins. In contrast to this negative SHP-2 signaling, the role of SHP-2 as a positive regulator of TKR cell proliferation has been described. It has been suggested that SHP-2 plays a role in insulin-like growth factor-1- and other growth factor-stimulated cell proliferation through MAPK activation, but SHP-2 substrates linking SHP-2 to MAPK activation have not been identified, although it is probably located upstream of Ras (22, 45–47).

In analogy with the above-described model of the negative role of SHP-2 in TKR signaling, we propose that the B2 receptor, a novel ITIM-bearing receptor, can activate SHP-2, which subsequently dephosphorylates serum-activated TKR and/or downstream effectors. The resulting effect is inhibition of TKR-induced cell proliferation. This hypothesis is supported by this study and by the observation that bradykinin induces epidermal growth factor receptor (16) and insulin-like growth factor-1 re-
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