Targeting of PYK2 to Focal Adhesions as a Cellular Mechanism for Convergence between Integrins and G Protein-coupled Receptor Signaling Cascades*

Received for publication, May 17, 2000, and in revised form, July 17, 2000
Published, JBC Papers in Press, July 27, 2000, DOI 10.1074/jbc.M004290200

Vladimir Litvak, Donghua Tian, Yoav David Shaul, and Sima Lev‡
From the Department of Neurobiology, Weizmann Institute of Science, 76100 Rehovot, Israel

The non-receptor tyrosine kinase PYK2 appears to function at a point of convergence of integrins and certain G protein-coupled receptor (GPCR) signaling cascades. In this study, we provide evidence that translocation of PYK2 to focal adhesions is triggered both by cell adhesion to extracellular matrix proteins and by activation of the histamine GPCR. By using different mutants of PYK2 as green fluorescent fusion proteins, we show that the translocation of PYK2 to focal adhesions is not dependent on its catalytic activity but rather is mediated by its carboxyl-terminal domain. Translocation of PYK2 to focal adhesions was attributed to enhanced tyrosine phosphorylation of PYK2 and its association with the focal adhesion proteins paxillin and p130Cas. Translocation of PYK2 to focal adhesions, as well as its tyrosine phosphorylation in response to histamine treatment, was abolished in the presence of a protein kinase C inhibitors or cytochalasin D treatment, whereas activation of protein kinase C by phorbol ester resulted in focal adhesion targeting of PYK2 and its tyrosine phosphorylation in an integrin-clustering dependent manner. Overexpression of a wild-type PYK2 enhanced ERK activation in response to histamine, whereas a kinase-deficient mutant substantially inhibited this response. Furthermore, inhibition of PYK2 translocation to focal adhesions abolished ERK activation in response to histamine treatment. These results suggest that PYK2 apparently links between GPCRs and focal adhesion-dependent ERK activation and can provide the molecular basis underlying PYK2 function at a point of convergence between signaling pathways triggered by extracellular matrix proteins and certain GPCR agonists.

Many G protein-coupled receptors (GPCRs) elicit mitogenic responses through activation of the Ras mitogen-activated protein kinase (MAPK) signaling cascade (1, 2). It is now evident that tyrosine kinases play an important role in this process (3, 4). Stimulation of various GPCRs induces a rapid tyrosine phosphorylation of many signaling proteins, including several protein tyrosine kinases. Tyrosine phosphorylation of the Grb2-interacting proteins Shc and Gab1 was shown to be induced upon LPA (5, 6), endothelin-1 (7), bradykinin (8), or thrombin stimulation (9). Likewise, receptor tyrosine kinases, such as epidermal growth factor or platelet-derived growth factor receptors, become tyrosine-phosphorylated in response to certain GPCR agonists including endothelin-1, LPA, and thrombin (10). Activation of these receptor tyrosine kinases induces the formation of complex between the Grb2 adaptor protein and the guanine nucleotide exchanger factor Sos, which upon recruitment to the plasma membrane allows activation of the small GTP-binding protein Ras and subsequent activation of the MAPK pathway (11). Among the non-receptor tyrosine kinases, the Src family members were suggested to link GPCR stimulation to MAPK pathway activation. Inhibition of Src activity significantly attenuated MAPK activation in response to LPA, bradykinin, or thrombin (12). Similarly, overexpression of Csk, a negative regulator of Src, inhibited the transactivation of epidermal growth factor receptor in response to LPA or d2-adrenergic receptor activation (13) and attenuated MAPK activation in response to bradykinin (14). Although it is not yet clear how GPCRs activate Src, the non-receptor tyrosine kinases focal adhesion kinase (FAK) and PYK2 may participate in this process. The major autophosphorylation site of FAK or PYK2 provides a binding site for the SH2 domain of Src. Binding of Src to tyrosine-phosphorylated PYK2 or FAK enhances Src tyrosine kinase activity, which in turn phosphorylates PYK2 and FAK on specific tyrosine residues, and probably also phosphorylates additional signaling molecules such as Shc (14, 15).

In many cell types, activation of Gαi or Gαq-coupled receptors leads to tyrosine phosphorylation of FAK and/or its most closely related kinase, PYK2 (8, 14, 16–19). Stimulation of LPA, bradykinin, endothelin-1, thrombin, or the P2Y2 receptor induces a rapid tyrosine phosphorylation of PYK2 (8, 14, 20–22). In many cases, enhanced tyrosine phosphorylation of PYK2 is concomitant with extracellular signal-regulated protein kinase (ERK) activation. We and others have previously shown that PYK2 plays an important role in MAPK signaling cascades mediated by elevation of intracellular calcium concentration (8) or by activation of GPCRs (14, 20). Activation of PYK2 by the GPCRs bradykinin or LPA stimulates ERK activation by a mechanism involving PYK2 autophosphorylation, association with the tyrosine kinase Src, recruitment of the Grb2-Sos complex, and subsequent activation of the Ras-MAP kinase signaling pathway (14). In addition to GPCRs, PYK2 is activated upon cell adhesion to the extracellular matrix (ECM)
Regulation of PYK2 Translocation to Focal Adhesions

PYK2 function at a point of convergence between signaling ERK activation and can provide the molecular basis underlying histamine stimulation. These results suggest that PYK2 activation-dependent mechanism, is required for PYK2 translocation, mediated either by binding of ECM proteins or by a PKC-specific inhibitors were used: for PKC-α the myriostoylated pseudosubstrate (Val-Glu-Ala-Arg-Thr-Glu-Ala-Leu-Arg-Gln) (Biomol, catalog number P-219) was used; for PKC-ε the octapeptide derived from the RACK-binding site for PKC-ε (Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) was used; and the scrambled analog (Leu-Ser-Glu-Thr-Lys-Pro-Ala-Val) (kindly provided by Dr. R. Neshler, Hadassah Medical Center, Hebrew University, Israel) was used as a control (37). The PKC-ε inhibitor and the scrambled analog were introduced into the cells by either Alexa donkey anti-mouse or with Cy3-conjugated goat anti-rabbit IgG, or both, as indicated. The specimens were analyzed by Zeiss LSM software time series function. 

RESULTS

Integrins and Histamine-induced Translocation of PYK2 to Focal Adhesions—The non-receptor tyrosine kinase PYK2 is activated by a variety of extracellular stimuli including cell adhesion to ECM proteins and GPCR agonists. It has therefore been suggested to function at a point of convergence of integrin and certain GPCR signaling cascades. To explore this hypothesis experimentally, we characterized the tyrosine phosphoryl-

and is localized to focal contacts in certain cell types (23–25). It has therefore been suggested to provide a link between GPCRs and focal adhesion-dependent ERK activation (2).

There are striking similarities between the tyrosine kinase PYK2 and FAK, including their structural organization (8, 23, 26), their sequence homology, their phosphorylation sites (27, 28), their activation by integrins, their dependence on actin filaments integrity, and their association with the focal adhesion proteins paxillin, p130Cas, and the Rac/Cdc42 GTase-activating protein Graf (29–31). This similarity may explain the ability of PYK2 to compensate for part of the functions of FAK in FAK-null cells (32). Nonetheless, PYK2 has other properties that distinguish it from FAK. PYK2 expression is more restricted than the nearly ubiquitous expression of FAK. Moreover, FAK and PYK2 are differentially activated and associate with distinct intracellular proteins. Several proteins have been shown to be exclusively associated with PYK2, including the ARF-GAP protein PAP (33) and the Nirs, a newly discovered family of proteins (34). In addition, activation of ERK in response to various extracellular stimuli appears to be more tightly regulated by PYK2, as compared with FAK (19).

In the present study, we provide evidence that the targeting of PYK2 to focal adhesions is induced by integrins, protein kinase C (PKC), and histamine receptor activation. Translocation of PYK2 to focal adhesions is attributed to an enhanced tyrosine phosphorylation of PYK2 and its association with the focal adhesion proteins paxillin and p130Cas. Integrin clustering, mediated either by binding of ECM proteins or by a PKC activation-dependent mechanism, is required for PYK2 translocation to focal adhesions. In addition, targeting of PYK2 to focal adhesions is required for ERK activation in response to histamine stimulation. These results suggest that PYK2 apparently links between GPCRs and focal adhesion-dependent ERK activation and can provide the molecular basis underlying PYK2 function at a point of convergence between signaling pathways triggered by ECM proteins and certain GPCR agonists.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies against paxillin and p130Cas were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies against vinculin and phosphorylated ERK2 were from Santa Cruz Biotechnology (Santa Cruz, CA). The mammalian expression vectors pEGFP-N1 and pEGFP-C2 were purchased from Clontech Laboratories Inc. and were indicated, fibronectin (20 μg/ml)-coated latex beads as described previously by (37). The PKC-ε inhibitor and the scrambled analog were introduced into the cells by either Alexa donkey anti-mouse or with Cy3-conjugated goat anti-rabbit IgG, or both, as indicated. The specimens were analyzed by Zeiss LSM software time series function. 

Immuno, Immunoblotting, and Cell Stimulation—HeLa cells were seeded into 100-mm diameter dishes in DMEM containing 10% fetal bovine serum. Two days later, the cells were serum-starved in DMEM containing 0.1% fetal bovine serum for 24 h and then washed in PBS containing 0.5 mM EGTA and 0.75 mM MgCl2, 0.1 mM Na3VO4, 1 mM NaF, 0.1 mM sodium orthovanadate, and then kept in suspension for 1 h at 37 °C in DMEM containing 1 mg/ml BSA and 20 mM Hepes, pH 7.4. The cells were then seeded either on fibronectin (20 μg/ml) or poly-l-lysine (20 μg/ml)-coated dishes as described previously (35). The cells were allowed to attach and spread for different times as indicated and then washed with ice-cold PBS, lysed in lysis buffer containing 0.1% Triton X-100, 0.15 M NaCl, 5 mM Hepes, pH 7.4, 1 mM EDTA, 20 mM NaF, 0.75 mM MgCl2, 0.1 mM Na3VO4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, and immunoprecipitated as described previously (34). Immunoprecipitations and immunoblotting were performed as described previously (34). 

Indirect Immunofluorescence—HeLa cells were seeded on glass coverslips placed in 24-well dishes at a density of 2 × 104 cells/well. Where indicated, fibronectin (20 μg/ml)-coated coverslips were used. The cells were rinsed with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then incubated for 30 min in blocking buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 2% BSA, 1% glycine, 10% goat serum, and 0.1% Triton X-100). After 1 h of incubation with various primary antibodies, the cells were washed with PBS and incubated either with Alexa donkey anti-mouse or with Cy3-conjugated goat anti-rabbit IgG, or both, as indicated. The specimens were analyzed by Zeiss LSM-510 laser scanning confocal microscope at 488-nm and 543-nm excitations. In all cases 0.9-μm-thick images are shown. Imaging of GFP-PYK2 in Living Cells—A laser-scanning confocal microscope (Zeiss-LSM 510) was used to monitor the translocation of GFP fusion proteins in response to diverse stimuli. Cells expressing GFP fusion proteins, on 25-mm coverslips, were incubated at 37 °C in phenol-red free culture medium containing 20 μM Hepes and 1 mg/ml BSA. The various drugs were applied to the cells during the scanning of GFP-expressing cells. The cells were imaged using a Zeiss Plan-Apochromat (1.3 NA) × 40 objective, with a 488-nm laser line for excitation and 505-nm long-pass filter for emission. Fluorescent signals were collected sequentially using the Zeiss LSM software time series function.

RESULTS

Integrins and Histamine-induced Translocation of PYK2 to Focal Adhesions—The non-receptor tyrosine kinase PYK2 is activated by a variety of extracellular stimuli including cell adhesion to ECM proteins and GPCR agonists. It has therefore been suggested to function at a point of convergence of integrin and certain GPCR signaling cascades. To explore this hypothesis experimentally, we characterized the tyrosine phosphoryl-

...
Tyrosine phosphorylation of PYK2 in response to integrin or histamine receptor activation. A, serum-starved HeLa cells were subjected to replating assay as described under “Experimental Procedures.” PYK2 was immunoprecipitated (IP) either from the suspended cells (Susp.) or from cells that were attached to poly-L-lysine (PLL)- or fibronectin (FN)-coated dishes for the indicated lengths of time. PYK2 immunoprecipitates were analyzed by immunoblotting with either anti-phosphotyrosine (upper panel) or anti-PYK2 antibodies (lower panel). Serum-starved HeLa cells either were incubated with fibronectin- or poly-L-lysine-coated latex beads (B) or were stimulated with histamine (100 μM) (C) for the indicated lengths of time, and PYK2 immunoprecipitates were analyzed by Western blotting as described above.

To explore the subcellular localization of PYK2 in response to integrins or histamine receptor activation, we generated a series of constructs encoding enhanced GFP attached either to the full-length PYK2 (GFP-PYK2), to the kinase-deficient mutant PKM (GFP-PKM), or to the amino- or carboxyl-terminal domains of PYK2 as indicated. Tyrosine-phosphorylated GFP-PYK2 and GFP-PKM were shown in the right panel. Molecular weight markers are indicated. PYK2, lane 1; GFP-PYK2, lane 2; GFP-PKM, lane 3; GFP-PC, lane 4; and GFP-PN, lane 5.

Fig. 2. Expression of GFP-PYK2 fusion proteins. A, schematic structures of GFP-PYK2 fusion proteins. Shown are wild-type PYK2 fused to GFP (GFP-PYK2), the kinase-deficient mutant PKM (GFP-PKM), and the amino- and carboxyl-terminal domains (GFP-PN and GFP-PC), respectively. PR, proline-rich region; K, the conserved lysine within the ATP-binding site that was substituted by alanine in PKM. B, Western analysis of GFP-PYK2 fusion proteins. HEK 293 cells were transiently transfected with the different GFP-PYK2 constructs. The expression of the GFP-PYK2 fusion proteins was determined by immunoblotting using antibodies against the amino- or carboxyl-terminal domains of PYK2 as indicated. Tyrosine-phosphorylated GFP-PYK2 and GFP-PKM are shown in the right panel. Molecular weight markers are indicated. PYK2, lane 1; GFP-PYK2, lane 2; GFP-PKM, lane 3; GFP-PC, lane 4; and GFP-PN, lane 5.

The localization of GFP-PYK2 in living cells in response to integrin or histamine receptor activation was analyzed by confocal scanning laser microscopy. GFP or GFP-PYK2 were transiently transfected into HeLa cells and either subjected to replating assay on fibronectin-coated coverslips for 20 min at 37 °C or stimulated with histamine for 5 min at 37 °C. The confocal images shown in Fig. 3 demonstrate that similar to...
Fig. 3. GFP-PYK2 translocates to focal adhesions in response to integrin or histamine activation. A, shown are confocal micrographs of serum-starved HeLa cells expressing GFP or GFP-PYK2 either before cell detachment and 20 min after attachment to fibronectin (FN)-coated coverslips, or before histamine stimulation and 5 min after histamine treatment. B, GFP-PYK2, but not GFP, is colocalized with vinculin and paxillin at focal adhesions. Indirect immunofluorescence staining of FN-replated HeLa cells expressing GFP or GFP-PYK2 with anti-vinculin or anti-paxillin antibodies was carried out. Shown are confocal micrographs of GFP- or GFP-PYK2-expressing cells (green, middle panels), and of Cy3-labeled vinculin or paxillin (red, left panels). The merged images are shown in the right panels. Yellow color indicates overlap of GFP with Cy3 staining. Scale bar is 10 μm.
GFP, GFP-PYK2 was evenly distributed in the cytoplasm of serum-starved cells. Integrin or histamine receptor activation had no effect on the cytosolic localization of GFP but induced a striking translocation of GFP-PYK2 to focal adhesion-like structures. These adhesive structures are large integrin aggregates to which the actin cytoskeleton is tethered, and numerous signaling components are recruited (38, 39). Several structural proteins, such as vinculin and paxillin, are colocalized with integrins at focal adhesions. To confirm the localization of GFP-PYK2 at focal adhesions, an indirect immunofluorescence analysis was carried out by using anti-paxillin or anti-vinculin antibodies. The results shown in Fig. 3B demonstrate that GFP-PYK2 is colocalized with vinculin and paxillin at focal adhesions following integrin activation, whereas no detectable colocalization of GFP was observed under the same experimental conditions. Similar results were obtained in response to histamine treatment (data not shown).

**Translocation of PYK2 to Focal Adhesions Is Independent of PYK2 Catalytic Activity but Rather Is Mediated by Its Carboxyl-terminal Domain**—To determine whether PYK2 catalytic activity is necessary for its translocation to focal adhesions, we have used the GFP-PKM construct and analyzed its localization in response to integrin activation. The results shown in Fig. 4 demonstrate that similar to GFP-PYK2, GFP-PKM was translocated to focal adhesions. Although GFP-PKM-expressing cells usually exhibit round morphology and are easily distinguished from GFP-PYK2-expressing cells, we did not detect any effect of GFP-PKM on cell spreading or adhesion as demonstrated 5 min after replating (Fig. 4). However, 20 min later, GFP-PKM was already localized in focal adhesions, whereas GFP-PN was still distributed in the cytosol. These cellular localization of GFP-PKM and GFP-PN were maintained for at least 1 h. Furthermore, GFP-PKM was coimmunoprecipitated with paxillin following integrin or histamine activation (data not shown). These results indicate that translocation of PYK2 to focal adhesions is not dependent on its catalytic activity but rather is mediated by its carboxyl-terminal domain, probably through protein-protein interactions with focal adhesion proteins such as paxillin or p130Cas.

**Tyrosine Phosphorylation and Focal Adhesion Targeting of PYK2 in Response to Histamine Requires PKC Activation**—To determine the mechanism by which histamine induces translocation of PYK2 to focal adhesions, we first characterized the tyrosine phosphorylation of PYK2 in response to histamine treatment. Stimulation of HeLa cells with histamine leads to H1 receptor-mediated production of inositol trisphosphate and diacylglycerol and the subsequent mobilization of calcium from intracellular stores and activation of PKC (40). We therefore assessed the phosphorylation of PYK2 in response to histamine in the absence or presence of the highly selective PKC inhibitor, GF109203X (41). The results shown in Fig. 5A demonstrate that histamine induced strong tyrosine phosphorylation of PYK2, which was completely abolished in the presence of GF109203X, suggesting that PKC activation is required for tyrosine phosphorylation of PYK2 in response to histamine treatment. Similar inhibition was obtained either by prolonged treatment with PMA, which induces down-regulation of the PMA-sensitive PKC isozymes, or by pretreatment with cytochalasin D, which disrupts actin polymerization (42) (Fig. 5A).

To assess the necessity of PKC activation for the translocation of PYK2 to focal adhesions in response to histamine,
rum-starved HeLa cells expressing the GFP-PYK2 were pre-
treated with GF109203X, stimulated with histamine, and
analyzed by confocal scanning laser microscopy. The results
shown in Fig. 5B demonstrate that under these experimental
conditions, GFP-PYK2 was retained in the cytosol, and no
detectable translocation to focal adhesions was evident at any
time after histamine treatment. These results suggest that
activation of PKC is not only required for tyrosine phosphi-
lation of PYK2 but is also crucial for its translocation to focal
adhesions. Furthermore, disruption of integrin clustering at
focal adhesions as a result of cytochalasin D pretreatment
prevented PYK2 translocation in response to histamine (Fig.
5B).

**Targeting of PYK2 to Focal Adhesions by PKC Activation**—To evaluate the role of PKC activation on PYK2 translo-
cation, we determined its localization in response to phorbol
ester treatment. Serum-starved HeLa cells expressing GFP-
PYK2 were pretreated for 20 min at 37 °C with PMA and
analyzed by confocal scanning laser microscopy. As shown in
Fig. 6A, PMA induced a striking translocation of GFP-PYK2 to
focal adhesion-like structures. Pretreatment with either the
PKC inhibitor GF109203X or with cytochalasin D abolished the
translocation of GFP-PYK2 to focal adhesions in response to
PMA treatment, similar to the results obtained in response to
histamine treatment (Fig. 5B). This demonstrates that trans-
location of PYK2 to focal adhesions by PMA is mediated by the
activation of PKC, and it is dependent on integrin clustering in
focal adhesions. Since PMA is a very potent stimulant of PYK2
activity in many cell types, the translocation of PYK2 to focal
adhesions may provide a general mechanism by which PKC
activates PYK2.

We therefore characterized the tyrosine phosphorylation of
endogenous PYK2 in HeLa cells in response to PMA treatment.
Treatment of serum-starved HeLa cells with PMA resulted in a
strong tyrosine phosphorylation of PYK2. This phosphorylation
is mediated by PKC activation and is dependent on the integ-

Fig. 5. GF109203X and cytochalasin D inhibit tyrosine phosphorylation and focal adhesion, targeting of PYK2 in response to
histamine treatment. A, quiescent HeLa cells were incubated with histamine (100 μM) for 10 min at 37 °C. Where indicated, the cells were
pretreated either with cytochalasin D (5 μg/ml) (CytoD) for 30 min, GF109203X (2 μM) for 12 h, or with 200 nM PMA for 12 h to induce
down-regulation of PKC (PKC DR), before histamine stimulation. PYK2 was immunoprecipitated (IP) with anti-PYK2 antibodies, and the IPs were
resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either anti-phosphotyrosine (upper panel) or anti-PYK2 (lower
panel) antibodies. B, confocal images of serum-starved HeLa cells expressing GFP-PYK2 before and 5 min after histamine treatment. Where
indicated, the cells were pretreated with either cytochalasin D or GF109203X, as described above.
Association of PYK2 with Focal Adhesion Proteins in Response to Histamine Treatment—Several focal adhesion proteins have been shown previously to interact with PYK2 in response to integrin activation (25, 30). To assess whether translocation of PYK2 to focal adhesions can be attributed to an enhanced association with the focal adhesion proteins paxillin and p130Cas, we immunoprecipitated PYK2 following cell stimulation with histamine or PMA. PYK2 immunoprecipitates were resolved by SDS-PAGE, and the presence of paxillin or p130Cas in PYK2 immunocomplexes was determined by immunoblotting with the appropriate antibodies (Fig. 7A). By using the same approach, we showed that PYK2 is present in paxillin or p130Cas immunocomplexes in response to histamine or PMA treatment (Fig. 7B). Thus, histamine or PMA induce targeting of PYK2 to focal adhesions and enhance its tyrosine phosphorylation and its association with the focal adhesion proteins paxillin and p130Cas.

PYK2 Provides a Link between the Histamine Receptor and Focal Adhesion-dependent ERK Activation—To determine whether translocation of PYK2 to focal adhesions is required for histamine downstream signaling, we first characterized the effect of histamine on ERK activation in HeLa cells. Serum-starved HeLa cells were treated with histamine for different lengths of time, and ERK activation was determined by Western analysis using antibodies against the dually phosphorylated active form of ERK1/2. The results shown in Fig. 8A demonstrate that histamine induced rapid activation of ERK following cell stimulation. This activation was sustained for 15 min after histamine treatment and then declined. By contrast, activation of ERK in response to PMA was sustained for at least 30 min after PMA treatment (Fig. 8A).

To determine whether PYK2 is an upstream regulator of ERK activation in response to histamine treatment, HeLa cells were transiently transfected with HA-tagged ERK2, either alone or together with an expression construct encoding the wild-type PYK2 or the kinase-deficient mutant PKM. The cells were stimulated with histamine for 10 min at 37 °C, and ERK2-HA was immunoprecipitated with anti-HA antibodies, resolved by SDS-PAGE, and immunoblotted with antibodies against phosphorylated ERK1/2. As shown in Fig. 8B, histamine induced strong activation of ERK2, which was further enhanced by overexpression of wild-type PYK2, but significantly inhibited by overexpression of the kinase-deficient mutant PKM. These results suggest that PYK2 is an upstream regulator of ERK activation in response to histamine.
Regulation of PYK2 Translocation to Focal Adhesions

Since both GF109203X and cytochalasin D inhibit PYK2 translocation to focal adhesions in response to histamine treatment, and since ERK activation by histamine is mediated by PYK2 activation, we propose that PYK2 apparently provides a link between the histamine GPCR and focal adhesion-dependent ERK activation.

**DISCUSSION**

The non-receptor tyrosine kinase PYK2 is activated by a variety of extracellular stimuli, including cell adhesion to ECM proteins and GPCR agonists. It has therefore been suggested to function at a point of convergence of integrin and certain GPCR signaling cascades. In the present study, we provide evidence that PYK2 is tyrosine-phosphorylated in response to integrin and the histamine GPCR activation (Fig. 1). By using GFP-tagged PYK2, we show that these extracellular stimuli induce translocation of PYK2 to focal adhesions, where it is colocalized with the focal adhesion proteins vinculin and paxillin (Fig. 3). Translocation of PYK2 to focal adhesion is not dependent on PYK2 catalytic activity but rather is mediated by its carboxy-terminal domain (Fig. 4). The carboxy-terminal domain of PYK2 shares approximately 40% sequence identity with the carboxy-terminal domain of FAK. This domain contains a 140-amino acid sequence designated the “focal adhesion targeting” domain. The focal adhesion targeting domain was shown to be both necessary and sufficient for the targeting of FAK to focal contacts (44). PYK2 contains a putative focal adhesion targeting domain as well, but several reports have presented somewhat conflicting data regarding its cellular function. PYK2, when overexpressed in chicken fibroblasts, was mainly distributed in the cytosol, whereas the PYK2 carboxy-terminal domain was localized to focal adhesions (45). Similar results were obtained in mouse fibroblasts: PRK (a PYK2-related non-kinase), when expressed in Swiss 3T3 cells, was efficiently localized to focal adhesions (46), whereas the full-length PYK2 mainly accumulated in the cytoplasm and caused cell apoptosis (47). These studies suggest that the amino-terminal domain of PYK2 somehow interferes with focal adhesion localization of PYK2. In contrast to these reports, we show here that PYK2 displays an integrin-dependent phosphorylation and focal adhesion localization, which is consistent with previous studies demonstrating that PYK2 is activated in response to integrin engagement in different cell types, including B-cells (29), megakaryocytes (24), T-cells (48), natural killer cells (25), and others. The different cell types used in these studies, as well as the different subset of integrins that were activated, may account for these conflicting results. This hypothesis is strengthened by recent studies demonstrating the complexity and specificity of integrin signaling (49).

The targeting of PYK2 to focal adhesions in response to activation of histamine GPCR was inhibited by pretreatment with either PKC inhibitors or cytochalasin D (Fig. 5). These inhibitors also abolished the tyrosine phosphorylation of PYK2 in response to histamine, thus demonstrating that both the integrity of the actin cytoskeleton and activation of PKC are required for PYK2 translocation to focal adhesions, as well as for its tyrosine phosphorylation. The role of PKC activation in the targeting of PYK2 to focal adhesions was assessed in response to phorbol ester treatment. As observed for histamine, PKC activation induced translocation of PYK2 to focal adhesions in a PKC activation- and integrin-clustering-dependent manner. The PKC inhibitor GF109203X, and cytochalasin D, which disrupts integrin clustering in focal adhesions, prevented PYK2 translocation in response to PMA treatment (Fig. 6A).

PKC appears to be one of the key intermediates in integrin-mediated signaling and was shown to induce integrin clustering in many cell types (50). In certain cells, inhibition of PKC

---

**Fig. 7. Association of PYK2 with p130Cas and paxillin in response to histamine and PMA treatment.** A, quiescent HeLa cells were stimulated with either histamine or PMA as indicated; PYK2 was immunoprecipitated (IP) by anti-PYK2 antibodies, and the immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies against p130Cas, paxillin, PTYR, or PYK2, as indicated. B, quiescent HeLa cells were stimulated as described above; p130Cas and paxillin were immunoprecipitated by anti-p130Cas or anti-paxillin antibodies, and the presence of PYK2 in their immunocomplexes was determined by immunoblotting with anti-PYK2 antibodies.

---

**Table 1: Regulation of PYK2 Translocation to Focal Adhesions.**

| Stimulus | PYK2 Translocation |
|----------|-------------------|
| Histamine | Yes |
| PMA | Yes |
| GF109203X | No |
| Cytochalasin D | No |

---

**References:**

1. Doe, J. and Smith, D. (2023). Regulation of PYK2 Translocation to Focal Adhesions. Cell 154, 1234-1247.
2. Doe et al. (2023). Regulation of PYK2 Translocation to Focal Adhesions. Cell 154, 1234-1247.
3. Doe et al. (2023). Regulation of PYK2 Translocation to Focal Adhesions. Cell 154, 1234-1247.
4. Doe et al. (2023). Regulation of PYK2 Translocation to Focal Adhesions. Cell 154, 1234-1247.
5. Doe et al. (2023). Regulation of PYK2 Translocation to Focal Adhesions. Cell 154, 1234-1247.
activity prevents focal adhesion formation, cell attachment, and cell spreading (51, 52). In HeLa cells, activation of PKC led to rapid and robust cell spreading, as well as tyrosine phosphorylation of PYK2 (Fig. 6, A and B). Pretreatment with GF109203X or down-regulation of the PMA-sensitive PKC isozymes abolished the tyrosine phosphorylation of PYK2 in response to PMA, thus demonstrating that the effect of PMA on PYK2 tyrosine phosphorylation is mediated by PKC activation. In addition, disruption of integrin clustering in focal adhesions as a result of cytochalasin D pretreatment abolished the tyrosine phosphorylation of PYK2 in response to PMA (Fig. 6B). These results demonstrate that the translocation of PYK2 to focal adhesions in response to PMA coincides with an increase in its tyrosine phosphorylation in a PKC activation-dependent manner.

The translocation of PYK2 to focal adhesions in response to histamine or PMA was attributed to an increase in its association with the focal adhesion proteins paxillin and p130Cas (Fig. 7). Thus, the targeting of PYK2 to focal adhesions can be triggered either by binding of ECM proteins (Fig. 3) or by a PKC activation-dependent mechanism (Figs. 5 and 6). The localization of PYK2 at focal adhesions may be stabilized by interaction with focal adhesion proteins such as paxillin or p130Cas, as illustrated in Fig. 9.

Although activation of integrin, histamine, or PKC induces targeting of PYK2 to focal adhesions, they differentially affect the dynamic trafficking of PYK2 to this subcellular location (our preliminary results). The effect of histamine on PYK2 trafficking is very rapid and already appears 30 s after histamine treatment. Real time imaging of GFP-PYK2 translocation in response to histamine revealed a highly dynamic movement to focal adhesion-like structures. This rapid traf-
flickering of PYK2 in response to histamine could result from local calcium concentrations. Since histamine evokes a repetitive increase in intracellular calcium ion concentration (Ca$^{2+}$ spikes) (40), it could be that the dynamic movement of PYK2 in response to histamine is regulated by calcium spiking, whereas its targeting to focal adhesions, as we show here, is mediated by PKC activation. We are currently investigating these possibilities.

We have previously shown that PYK2 acts as an upstream regulator of ERK activation in response to different cellular stimuli (8, 14). In HeLa cells histamine induced a rapid activation of ERK, which was substantially inhibited by overexpression of the kinase-deficient mutant PKM, and was significantly enhanced by overexpression of the wild-type PYK2. Activation of ERK by histamine was inhibited by down-regulation of PKC, by the PKC inhibitor GF109203X, and by cytochalasin D pretreatment (Fig. 8). Since these treatments abolished the translocation of PYK2 to focal adhesions (Figs. 5 and 6) and its tyrosine phosphorylation (Figs. 5 and 6), and since PYK2 is required for ERK activation by histamine (Fig. 8B), we propose that PYK2 apparently provides a link between the histamine GPCR and focal adhesion-dependent ERK activation. Furthermore, real time imaging of GFP-tagged PKC-ε, -β, -ε, and -γ trafficking in response to histamine treatment revealed that the two PKC isozymes ε and β translocate to the plasma membrane (data not shown). Inhibition of these isozymes by PKC isozyme-specific inhibitors attenuated the tyrosine phosphorylation of PYK2 as well as ERK activation in response to histamine treatment (Fig. 8D), thus demonstrating that the PKC-β and -ε probably mediate the effect of histamine on PYK2 tyrosine phosphorylation and ERK activation in HeLa cells.

Taken together, by integrating imaging techniques with biochemical and pharmacological studies, we show that the histamine GPCR induces translocation of PYK2 to focal adhesions, enhances PYK2 tyrosine phosphorylation, and activates ERK through a PYK2 translocation-dependent mechanism. Whether this is a general mechanism for activation of PYK2 by GPCRs or an exclusive mechanism used by the histamine GPCR remains to be determined.

Acknowledgments—We thank Shari Carmon for technical assistance, Eduard Korkotian for guidance with confocal microscopy, and Dr. R. Zeger for the anti-MAPKs antibodies. We thank C. Brodie and R. Nesher for the GFP-tagged PKC isozymes and the PKC isozyme-specific inhibitors. We also thank V. Teichberg and I. Nevo for critical reading of the manuscript.

REFERENCES

1. Outkind, J. S. (1998) J. Biol. Chem. 273, 1839–1842
2. Luttrell, L. M., Daaka, J., and Lefkowitz, R. J. (1999) Curr. Opin. Cell Biol. 11, 177–183
3. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Mooenlaar, W. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1257–1261
4. Sadowska, J., and Inamo, S. (1991) EMBO J. 15, 775–777
5. van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Toshara, K., Purift, E., Sakae, M., Luttrell, L. M., and Lefkowitz, R. J. (1995) Nature 376, 781–784
6. Daub, H., Wallach, C., Lankena, A., Herrlich, A., and Ulrich, A. (1997) EMBO J. 16, 7032–7044
7. Cazaubon, S. M., Ramos-Moraes, F., Fischer, S., Schwighofer, F., Struberg, D., and Couraud, P. O. (1998) Biochem. J. 330, 24805–24809
8. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) Nature 376, 737–745
9. Chen, Y., Graff, D., Salcini, A. E., Pelici, P. G., Pouyssegur, J., and Van Obbergen-Schilling, E. (1999) EMBO J. 15, 1037–1044
10. Daub, H., Weiss, F. U., Wallach, C., and Ulrich, A. (1996) Nature 379, 557–560
11. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) Nature 363, 45–51
12. Dia, W., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M. (1999) J. Biol. Chem. 274, 13978–13984
13. Hawes, B. E., van Biesen, T., Toshara, K., Luttrell, L. M., and Lefkowitz, R. J. (1995) J. Biol. Chem. 270, 17148–17153
14. Dikic, I., Takiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) Nature 383, 547–550
15. Slaperf, D. D., Hawks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–781
16. Simont-Smith, J., Zachary, L., Valverde, A. M., and Zon, E. R. (1993) J. Biol. Chem. 268, 8309–8315
17. Zachary, I., Simont-Smith, J., Turner, C. E., and Zon, E. R. (1993) J. Biol. Chem. 268, 14261–14268
18. Soontak, S. P., Avraham, H., Avraham, S., and Cantley, L. C. (1998) J. Biol. Chem. 273, 2515–2520
19. Soontak, S. P., Avraham, H., Avraham, S., and Cantley, L. C. (1998) J. Biol. Chem. 273, 2563–2566
20. Dia, W., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 19125–19132
21. Brinon, A. E., Harding, T., Dibbert, P. A., E., Li, Y., Hunter, D., Herman, B., and Graves, L. M. (1999) J. Biol. Chem. 274, 1711–1718
22. Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., and Sasaki, T. (1995) J. Biol. Chem. 270, 12156–12162
23. Li, J., Avraham, H., Rogers, R. A., Raja, S., and Avraham, S. (1996) Blood 147–148
24. Gissmondi, A., Bisogno, L., Mainiero, P., Palmieri, G., Piccoli, M., Frati, L., and Santoni, A. (1997) J. Immunol. 159, 4729–4736
25. Avraham, S., London, R., Fu, Y., Ota, S., Hirci, H., Law, S. F., Zeng, Y., Golemis, E. A., Fu, Y., Druker, B. J., Haghayeghi, N., Freedman, A. S., and Avraham, S. (1997) J. Biol. Chem. 272, 17919–17924
26. Lakakori, P. T., Nakamura, I., Nagy, E. M., Parsons, J. T., Rodan, G. A., and Duong, L. T. (1999) J. Biol. Chem. 274, 27242–27251
Regulation of PYK2 Translocation to Focal Adhesions

34. Lev, S., Hernandez, J., Martinez, R., Chen, A., Plowman, G., and Schlessinger, J. (1999) *Mol. Cell. Biol.* **19**, 2278–2788
35. Hanks, S. K., Calaph, M. B., Harper, M. C., and Patel, S. K. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8487–8491
36. Grinnell, F., and Geiger, B. (1986) *Exp. Cell Res.* **162**, 449–461
37. Yedovitzky, M., Mochly-Rosen, D., Johnson, J. A., Gray, M. O., Ron, D., Abramovitch, R., Cerasi, E., and Nesher, R. (1997) *J. Biol. Chem.* **272**, 1417–1420
38. Johnson, J. A., Gray, M. O, Karlins, G. S., Chen, C. H., and Mochly-Rosen, D. (1996) *Circ. Res.* **79**, 1086–1099
39. Hemler, M. E. (1998) *Curr. Opin. Cell Biol.* **10**, 578–585
40. Bootman, M. D., Young, K. W., Young, J. M., Moreton, R. B., and Berridge, M. J. (1996) *Biochem. J.* **314**, 347–354
41. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Daahamel, L., Charon, D., and Kirilovsky, J. (1991) *J. Biol. Chem.* **266**, 15711–15781
42. Rodriguez-Fernandez, J. L. (1999) *BioEssays* **21**, 1069–1075
43. Dekker, L. V., and Parker, J. P. (1994) *Trends Biochem. Sci.* **19**, 73–77
44. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1993) *J. Cell Biol.* **123**, 993–1005
45. Schaller, M. D., and Sasaki, T. (1997) *J. Biol. Chem.* **272**, 25319–25325
46. Xiong, W., Macklem, M., and Parsons, J. T. (1998) *J. Cell Sci.* **111**, 1981–1991
47. Xiong, W., and Parsons, J. T. (1997) *J. Cell Biol.* **139**, 529–539
48. Ma, E. A., Lou, O., Berg, N. N., and Ostergaard, H. L. (1997) *Eur. J. Immunol.* **27**, 329–335
49. Giancotti, F. G. (2000) *Nat. Cell Biol.* **2**, E13–E14
50. Sanchez-Mateos, P., Cabanas, C., and Sanchez-Madrid, F. (1996) *Semin. Cancer Biol.* **7**, 99–109
51. Wood, A., and Couchman, J. R. (1992) *J. Cell Sci.* **101**, 277–290
52. Haimovich, B., Kaneshiki, N., and Ji, P. (1996) *Blood* **87**, 152–161