Protein-tyrosine Kinase Pyk2 Mediates Endothelin-induced p38 MAPK Activation in Glomerular Mesangial Cells*

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Endothelin-1 (ET-1), a family of a member of 21 amino acid peptides possessing vasoconstrictor properties, is known to stimulate mesangial cell proliferation. In this study, ET-1 (100 nM) induced a rapid activation of p21WBS in human glomerular mesangial cells (HMC). Inhibition of Src family tyrosine kinase activity with [4-Amino-5-(4-methylphenyl)-7-(t-buty)pyrazolo[3,4-d]pyrimidine] or chelation of intracellular free calcium with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester significantly decreased ET-1-dependent p21WBS activation and suggested the involvement of the cytoplasmic proline-rich tyrosine kinase Pyk2. We have observed that Pyk2 was expressed in HMC and was tyrosine-phosphorylated within 5 min of ET-1 treatment. ET-1-induced activation of Pyk2 was further confirmed using phospho-specific anti-Pyk2 antibodies. Surprisingly, Src kinase activity was required upstream of ET-1-induced autophosphorylation of Pyk2. To determine whether Pyk2 autophosphorylation mediated ET-1-dependent p21WBS activation, adenovirus-mediated transfer was employed to express a dominant-negative form of Pyk2 (CRNK). CRNK expression inhibited ET-1-induced endogenous Pyk2 autophosphorylation, but did not abolish ET-1-mediated increases in GTP-bound p21WBS levels. ET-1-induced activation of the p38 MAPK (but not ERK) pathway was inhibited in HMC and in rat glomerular mesangial cells expressing the dominant-negative form of Pyk2. These findings suggest that the engagement of Pyk2 is important for ET-1-mediated p38 MAPK activation and hence the biological effect of this peptide in mesangial cells.

The proliferation of glomerular mesangial cells is a key event in the development of proliferative inflammatory renal diseases (1). Endothelin-1 (ET-1)† is a member of a family of 21 amino acid peptides possessing potent vasoconstrictor properties whose ability to stimulate mesangial cell proliferation is well established (2). Moreover, ET-1, acting in concert with other vasoactive mediators, cytokines, and growth factors, plays an integral role in the pathogenesis of proliferative glomerulonephritis. Stimulation of mesangial cells with ET-1 evokes a wide variety of signaling events (for review, see Ref. 3); however, ET-1-induced cell proliferation occurs primarily via its activation of the intracellular mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK). The p38 group of MAPKs has been found to be involved in inflammation, cell contraction, and cell death (4, 5). Contractile responsiveness of mesangial cells was shown to depend on activation of the p38 MAPKs (5, 6) and could perhaps partially account for ET-1 contractive properties.

On the basis of the genetic and biochemical evidence, it seems likely that p21WBS, a member of the small GTPase superfamily, has a crucial role in growth factor-induced stimulation of ERK and resultant renal cell proliferation (for review, see Ref. 7). The active form of p21WBS is bound to GTP, whereas the inactive form is bound to GDP. The exchange of GTP for GDP is promoted by guanosine nucleotide exchange factors, which are recruited to the signaling complex by adaptor proteins (for review, see Ref. 8). Recent findings suggest that ET-1-dependent p21WBS activation in mesangial cells requires tyrosine phosphorylation of the adaptor protein Shc and the subsequent formation of the Shc-Grb2-Sos signaling complex (9). The precise mechanism by which ET-1 induces Shc phosphorylation has not been defined. In analogy to p21WBS regulation of ERK, members of the Rho family of small GTPases are positive regulators of p38 MAPK pathways (10). However, the exact mechanisms of ET-1 stimulation of p38 MAPKs have not yet been clarified.

The actions of ET-1 are mediated by ligand-dependent activation of specific G protein-coupled receptors (GPCRs) (for review, see Ref. 11). GPCRs are devoid of intrinsic tyrosine kinase activity; therefore, the protein tyrosine phosphorylation induced by ligands of GPCRs depends ultimately upon subsequent activation of cellular tyrosine kinases. Evidence suggests that ET-1 activates members of the Src family of cytoplasmic tyrosine kinases (12–14). Tyrosine phosphorylation appears to be essential for the mitogenic effects of many GPCRs ligands, including ET-1 (3), so it is not surprising that the signaling pathways linking GPCR activation with mobilization of cellular tyrosine kinases have become the subject of intensive investigation.

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‡The abbreviations used are: ET-1, endothelin-1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; FAK, focal adhesion kinase; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; PP1, [4-Amino-5-(4-methylphenyl)-7-(t-buty)pyrazolo[3,4-d]pyrimidine]; HMC, human mesangial cells; m.o.i., multiplicity of infection; RBD, p21WBS-binding domain; GST, glutathione S-transferase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; PAGE, polyacrylamide gel electrophoresis; JNK, c-Jun N-terminal kinase; JAK, Janus kinase; STAT, signal transducers and activators of transcription; MAPKAPK, MAP kinase-activated protein kinase.
One of the typical cellular responses to ligand-dependent GPCR activation shared by ET-1 is mobilization of intracellular calcium (for review, see Ref. 15). The cloning of the calcium-regulated cytoplasmic proline-rich tyrosine kinase Pyk2 (also known as related adhesion focal tyrosine kinase (RAFTK), focal adhesion kinase-2 (FAK2), and cell adhesion kinase β (CAKβ), calcium-dependent tyrosine kinase (CADTK)) suggested the link between GPCRs and the induction of tyrosine phosphorylation via mobilization of intracellular calcium (16–20). Moreover, a number of studies have supported the role of Pyk2 in coupling GPCRs with MAPK activation (21, 22). ERK activation by ET-1 was found to coincide with Pyk2 tyrosine phosphorylation in primary astrocytes (13).

Pyk2 and FAK belong to a distinct family of cytoplasmic protein-tyrosine kinases that are regulated by extracellular stimuli (23). Although FAK and Pyk2 may have partially redundant roles, they also exhibit distinct differences, notably with regard to their substrate specificity (24–26). The proline-rich region of Pyk2 interacts with a number of SH3 (Src homology) domain-containing proteins, including the docking protein Crk-associated substrate (p130Cas) (27) and PAP (Pyk2 C terminus-associated protein). Activation of Pyk2, but not FAK, leads to tyrosine phosphorylation of PAP (26); furthermore, Pyk2 specifically phosphorylates the carboxyl-terminal cytosolic portion of the potassium channel Kv1.2 (28). Other proteins reported to associate with Pyk2 include paxillin (29, 30), leupaxin (31), Hic-5 (32), and the product of the Ewing sarcoma gene (EWS) (33).

In this study, we show that glomerular mesangial cells express significant amounts of Pyk2 and elucidate the role of calcium-regulated Pyk2 in mediating ET-1-induced signaling events in these cells. We demonstrate that (i) ET-1-mediated activation of p21ras depends upon mobilization of intracellular calcium and activation of Src family of kinases and that (ii) Pyk2 is tyrosine-phosphorylated in response to ET-1 and that adenosivirus-mediated transfer of a dominant interfering Pyk2 construct abolishes autophosphorylation of endogenous Pyk2, preventing ET-1-stimulated p38 MAPK activation. ET-1-mediated activation of p21ras and ERK activation were not abolished by adenosivirus-mediated transfer of a dominant interfering Pyk2 construct. Taken together, these findings suggest that engagement of the calcium-regulated protein-tyrosine kinase Pyk2 is important for cellular responses to ET-1 and hence the biological effects of this peptide.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture media and reagents were from Life Technologies, Inc. and BioWhittaker, Inc. (Walkersville, MD). Purified human ET-1, BAPTA/AM, PP2, and PP3 were from Calbiochem-Novabiochem. PPI was from Alexis Corp. (San Diego, CA). ECL reagent was supplied by Amersham Pharmacia Biotech (Little Chalfont, United Kingdom). The BCA protein assay kit was from Pierce. Bisindolylmaleimide I was from the protein kinase C inhibitor set from Calbiochem-Novabiochem. All other reagents were from Sigma.

**Antibodies**—Mouse monoclonal anti-human Ras (H-a-Ras) and anti-human Pyk2 antibodies were from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-Grb2, anti-p38 MAPK, and anti-FLAG antibodies and mouse monoclonal anti-Myc antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK1/2 antibodies were raised by immunizing rabbits with synthetic peptides and were described previously (34). Phosphorylation state-specific anti-Pyk2 Tyr-402, Tyr-579, Tyr-580, and Tyr-881 antibodies were from BIOSOURCE International (Camarillo, CA). Phosphorylation state-specific anti-ERK1/2 and phosphorylation state-specific anti-p38 MAPK antibodies were from New England Biolabs, Inc. (Beverly, MA). Horseradish peroxidase-conjugated anti-Myc antibodies (clone 9E10) were from Roche Molecular Biochemicals.

**Cells**—SV40-transformed human mesangial cells (HMC) were kindly provided by Jean-Daniel Sraer (INSERM Unite 64, Hopital Tenon, Paris, France) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 μM HEPES, 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (35). Primary rat glomerular mesangial cells (RMC) were cultured in RPMI 1640 medium supplemented with 17% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, 5 μg/ml each insulin and transferrin, and 5 ng/ml G418. RMC were passed between passages 5 and 8. Enzyme-linked immunosorbent assay was performed exactly as described previously (9). Cell lysis, immunoprecipitation of samples standardized for protein content, and Western blotting were performed as described previously (9).

**RESULTS**

**Endothelin-induced ras Activation in Human Mesangial Cells Depends on Calcium Mobilization and Activation of Src Family Kinases**—We have described previously the kinetics of ET-1-stimulated p21ras activation in glomerular mesangial cells (9). In RMC, ET-1 evokes a biphasic GTP loading of p21ras, with the first peak of activation occurring at 2–5 min and resulting in a monophasic activation of ERK1 and ERK2. It is generally accepted that GTP-bound p21ras is able to interact with Raf1, leading to activation of the ERK signaling cascade. We have previously demonstrated that p21ras activation is mediated by an immediate association of tyrosine-phosphorylated Shc with the guanosine exchange factor Sos1 via the adaptor protein Grb2. However, thus far, the identity of the tyrosine kinase responsible for phosphorylating Shc, leading to the resultant activation of p21ras, is undetermined. In this study, p21ras activation was assessed by means of an affinity binding assay that evaluates the quantity of GTP-bound p21ras protein able to associate with a GST-RBD fusion protein (see “Experimental Procedures”). As shown in Fig. 1, ET-1 induced a transient activation of p21ras in HMC, with the activity of p21ras being detected as early as 1.5 min. Preincubation of HMC with PP1, a selective inhibitor of Src family tyrosine kinases, significantly decreased p21ras activation. In addition, chelation of intracellular free calcium with BAPTA/AM also inhibited ET-1-dependent GTP loading of Ras (Fig. 1). These data suggest that p21ras is activated by ET-1 via Src family kinases in a calcium-dependent manner.

We next examined whether PP1 had any effect upon ET-1-induced activation of ERK1 and ERK2. ET-1 induced transient activation of ERK1 and ERK2 as detected by phosphorylation state-specific anti-ERK antibodies; as shown in Fig. 2, data...
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Pyk2 Is Expressed in HMC and Is Autophosphorylated in ET-1-treated Cells—The requirement of intracellular calcium mobilization and Src family kinase activation for ET-1-induced p21^{ras} activity suggested the involvement of the proline-rich tyrosine kinase Pyk2 in this signaling pathway. Pyk2 is a cytoplasmic tyrosine kinase known to be tightly regulated by intracellular free calcium levels and to interact with Src tyrosine kinases. Pyk2 expression has been reported to be restricted predominately to hematopoietic cells and cells of the nervous system (40). A number of studies have demonstrated Pyk2 expression in additional cell types (29, 41–43); therefore, to determine whether Pyk2 is expressed in HMC and whether it is activated in response to ET-1, HMC lysates were immunoprecipitated with anti-Pyk2 antibodies, followed by Western blot analysis using anti-phosphotyrosine antibodies and anti-Pyk2 antibodies (Fig. 3). Our results indicate that Pyk2 is expressed in HMC and that this protein is tyrosine-phosphorylated within 5 min of ET-1 treatment. ET-1-mediated Pyk2 activation was also detected by direct measurement of kinase-autophosphorylating activity in Pyk2 immunoprecipitates from HMC treated with ET-1 (data not shown).

To study ET-1-mediated Pyk2 tyrosine phosphorylation in greater details, we used phospho-specific anti-Pyk2 antibodies, which recognize phosphorylation of specific tyrosines residues, viz., Tyr-402, Tyr-579, Tyr-580, and Tyr-881. Phosphorylation of Tyr-579 and Tyr-580 is required for maximal kinase activity of Pyk2. Tyr-402 is an autophosphorylation site that can serve as a target for the Src SH2 domain, and phosphorylated tyrosine 881 was shown to interact with the Grb2 SH2 domain (22). As shown in Fig. 4, Tyr-402, Tyr-580, and Tyr-881 all were phosphorylated 4 min following ET-1 stimulation. Phosphorylation of Tyr-579 and Tyr-580 was found to be regulated similarly under all experimental conditions; therefore, data for only one of latter is shown.

We next investigated which tyrosine residues on Pyk2 required intracellular calcium mobilization for effective phosphorylation. As expected, ET-1-induced phosphorylation of all Pyk2 tyrosines was significantly attenuated in the presence of the calcium chelator BAPTA/AM (Fig. 4). The dramatic decrease in the extent of tyrosine phosphorylation observed in the presence of BAPTA/AM provides additional evidence for the selectivity of the phospho-specific antibodies used in this study since Pyk2 is unique among tyrosine kinases thus far described in its calcium-dependent activation. The identity of the band recognized by phospho-specific anti-Pyk2 antibodies was further confirmed by immunoprecipitation of Pyk2 with monoclonal anti-Pyk2 antibodies and immunoblotting either with phospho-specific anti-Pyk2 antibodies or with the same monoclonal anti-Pyk2 antibodies (data not shown).

Activation of Src Family Kinases Is Necessary for Signaling through Pyk2 in HMC—Our finding that Pyk2 Tyr-402 was autophosphorylated in ET-1-treated HMC raised the possibility that Pyk2 was associated with the tyrosine kinase c-Src. It has been shown that, following phosphorylation of a conserved C-terminal tyrosine residue in Src family protein kinases, the intramolecular interaction between this residue and the SH2 domain maintains these proteins in an inactive "closed" conformation (for review, see Ref. 40 and 44). Pyk2 and Src are associated via a reciprocal interaction between the phosphorylated Tyr-402 of the former and the SH2 domain of the latter. The formation of this complex disrupts the inhibitory intramo-

**Fig. 1. Inhibition of ET-1-mediated p21^{ras} activation by BAPTA/AM and PP1.** Quiescent HMC were preincubated either with BAPTA/AM (50 μM, 60 min) or with PP1 (50 μM, 10 min) and then stimulated with ET-1 (100 nM) for the times indicated. GTP-bound p21^{ras} was isolated by affinity precipitation with a GST-RBD fusion protein, followed by immunoblotting (IB) analysis with anti-p21^{ras} antibody (upper panel). The fusion protein was detected by Ponceau S staining of the nitrocellulose (lower panel). The positions of p21^{ras} and GST-RBD are indicated. Shown is a representative result; the experiment was repeated four times.

**Fig. 2. Inhibition of ET-1-induced ERK activation by PP1.** Quiescent HMC were preincubated with PP1 (50 μM, 10 min) and then stimulated with ET-1 (100 nM) for the times indicated. Cell lysates were analyzed using phospho-specific anti-ERK antibodies. The positions of ERK1 and ERK2 are indicated. Shown is a representative result; the experiment was repeated three times. IB, immunoblot.

**Fig. 3. Pyk2 is tyrosine-phosphorylated in response to ET-1.** Quiescent HMC were stimulated with ET-1 (100 nM) for the times indicated. Lysates were immunoprecipitated (IP) with a monoclonal anti-Pyk2 antibody. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting (IB) with anti-phosphotyrosine (anti-p-Tyr, upper panel) and anti-Pyk2 (lower panel) antibodies. The position of Pyk2 is indicated. Shown is a representative result; the experiment was repeated four times.
lecular interaction, thereby converting Src into its active form. We have coprecipitated Src with Pyk2 in ET-1-treated HMC and have also observed colocalization of Src with Pyk2 in ET-1-treated HMC by means of confocal microscopy (data not shown). Surprisingly, preincubation of these cells with PP1 or PP2 (selective inhibitors of Src family kinases) inhibited ET-1-induced tyrosine phosphorylation of Pyk2. As shown in Fig. 5, phosphorylation of Tyr-402 and Tyr-580 on Pyk2 in response to ET-1 was attenuated by PP2. This observed requirement for Src activity upstream of Pyk2 tyrosine phosphorylation was unexpected and indicated that an additional mechanism of Src activation was involved in ET-1-stimulated HMC.

It is known that activation of protein kinase C by the lipophilic second messenger sn-1,2-diacylglycerol contributes to mitogenic signaling by ET-1 in mesangial cells (45). Protein kinase C can directly activate c-Src by phosphorylation of Ser-12 and Ser-44 (46); therefore, we investigated the possibility that Pyk2 phosphorylation could occur via a protein kinase C-dependent pathway in ET-1-treated HMC. It was previously shown that depletion of protein kinase C activity partially inhibited both the angiotensin II- and platelet-derived growth factor-induced Pyk2 tyrosine phosphorylation (47). Phorbol ester (phorbol 12-myristate 13-acetate) was found to stimulate tyrosine phosphorylation of Pyk2 in HMC. Significant tyrosine phosphorylation of Pyk2 Tyr-402 and Tyr-580 was detected (Fig. 6). Furthermore, the phorbol 12-myristate 13-acetate-dependent effect was completely abolished by preincubation of HMC with PP2, but not with PP3, its inactive analog. In addition, preincubation of HMC with GF 109203X (bisindolylmaleimide; an inhibitor of protein kinase C) for 1.5 h decreased ET-1-induced autophosphorylation of Pyk2 as detected by Western blotting with phosphorylation state-specific anti-Pyk2 antibodies (data not shown). Taken together, these data suggest that both calcium mobilization and stimulation of Src family kinases (possibly in a protein kinase C-dependent manner) are required for ET-1-mediated Pyk2 activation.

Autophosphorylation of Pyk2 Mediates ET-1-induced Activation of p38 MAPK, but Not Activation of ERK and p21WAF1/CIP1. To determine whether Pyk2 autophosphorylation plays a role in p21WAF1/CIP1 activation by ET-1, we used adenovirus-mediated transfer to express Myc-tagged wild-type Pyk2 (Ad Pyk2 WT) and a FLAG-tagged dominant-negative form of Pyk2 (Ad Pyk2 CRNK) (Fig. 7A). CRNK represents a potential alternative spliced product of Pyk2 (24) that is able to inhibit autophosphorylation of endogenous Pyk2, but that has no effect upon tyrosine phosphorylation of FAK (36). A transgenic adenovirus encoding bacterial β-galactosidase activity (Ad LacZ) was used to routinely assess the efficiency of adenovirus-mediated gene transfer into HMC (Fig. 7B). Serum-starved HMC transduced with Ad Pyk2 WT, Ad Pyk2 CRNK, or Ad LacZ (70 plaque-forming units/cell) were stimulated with ET-1 for 4 min, lysed, resolved by SDS-PAGE, and immunoblotted with anti-Myc and anti-FLAG antibodies (Fig. 7, C and D). As expected, expression of Myc-tagged Pyk2 was detectable in cells infected with Ad Pyk2 WT, whereas FLAG-tagged Pyk2 CRNK was expressed in Ad Pyk2 CRNK-infected cells. As assessed by X-gal staining, 90% of cells in this experiment were infected (Fig. 7B).

To test the effect of wild-type Pyk2 and Pyk2 CRNK overexpression upon ET-1-stimulated Pyk2 autophosphorylation, the lysates of control or ET-1-stimulated (4 min) HMC were resolved by SDS-PAGE and immunoblotted with phospho-specific anti-Pyk2 antibodies. Adenovirus-mediated transfer of wild-type Pyk2 into HMC resulted in constitutive phosphorylation of Pyk2 Tyr-402, Tyr-580, and Tyr-881 (Fig. 8). The Pyk2 band detected by phosphorylation state-specific anti-Pyk2 antibodies in lanes corresponding to cells infected with Ad Pyk2 WT contained both endogenous Pyk2 and overexpressed Pyk2. ET-1 stimulated the phosphorylation of Pyk2 Tyr-402, Tyr-580, and Tyr-881 in Ad LacZ-infected cells, but did not have any additional effect on the autophosphorylation status of Pyk2 in cells infected with Ad Pyk2 WT. These data are consistent with results obtained with wild-type Pyk2 in other cell systems (36). HMC infected with Ad Pyk2 CRNK exhibited a lower level of endogenous Pyk2 autophosphorylation of Tyr-402 and Tyr-580 in response to ET-1. The overexpressed Pyk2 CRNK construct can be easily distinguished from endogenous Pyk2 due to its different mobility on SDS-polyacrylamide gel (Fig. 7). Surprisingly, Western blot analysis with phosphorylation state-specific anti-ERK1/2 antibodies did not reveal a decrease in the ability of ET-1 to induce ERK phosphorylation (Fig. 8).
We next checked whether ET-1-mediated activation of ERK1/2 would be inhibited when autophosphorylation of endogenous Pyk2 was completely abolished. At an infective ratio of 90 plaque-forming units/cell, a m.o.i. of 70 for 24 h, B, as assessed by X-gal staining, Ad LacZ infected 90% of the cells in this experiment. Lysates from Ad LacZ-, Ad Pyk2 WT-, and Ad Pyk2 CRNK-infected cells, quiescent or stimulated with ET-1 (100 nM) for 4 min, were resolved by SDS-PAGE and immuno-blotted with phospho-specific anti-Pyk2 Tyr-402 (pY402, first panel), Tyr-580 (pY580, second panel), and Tyr-881 (pY881, third panel) antibodies. The nitrocellulose was also probed with phosphorylation state-specific anti-ERK1/2 antibodies (anti-p-Erk, fourth panel) and with anti-Grb2 antibodies (fifth panel) to confirm equivalency of loading.

To verify our finding that dominant-negative Pyk2 interferes with the ability of ET-1 to activate the p38 MAPK signaling pathway, experiments were repeated in RMC. RMC display a higher level of dependence upon serum growth factors compared with HMC. The efficiency of adenovirus-mediated transfer of wild-type Pyk2 and the dominant-negative construct CRNK into RMC is demonstrated in Fig. 11. Western blotting with phosphorylation state-specific anti-p38 MAPK antibodies confirmed the inability of CRNK to prevent ET-1-induced ERK activation (Fig. 11, third panel). The ability of ET-1 to induce p38 MAPK activation was abolished by adenovirus-mediated transfer of the dominant-negative CRNK construct as detected by Western blotting with phosphorylation state-specific anti-p38 MAPK antibodies (Fig. 12).

**DISCUSSION**

Our data represent the first demonstration of the involvement of Pyk2 in ET-1 signaling in a non-neuronal cell type. Furthermore, we provide evidence that expression of the dominant interfering Pyk2 construct results in the inhibition of ET-1-mediated p38 MAPK activation.

ET-1 is a potent vasoconstrictor and is also a strong mitogen for a number of different cell types, including vascular smooth muscle cells, glomerular mesangial cells, and a number of fibroblast cell lines (2, 48). Activation of the cytoplasmic tyrosine kinase Pyk2 is a mechanism by which mobilization of intracellular calcium is coupled to stimulation of non-receptor tyrosine kinases in ET-1-treated cells. Since we have determined that ET-1-induced Pyk2 activation is linked to stimula-
tion of the p38 MAPK signaling pathway, it is possible that Pyk2 is one of the principal signaling molecules mediating the contractive properties of ET-1. It was reported that p38 MAPK mediates glomerular mesangial cell contraction (5). The ability of p38 MAPK to regulate cell contraction induced by GPCR ligands is possibly due to its unique capability to activate in vivo MAPKAP kinase-2/3 (49), which phosphorylates small HSP27. Small heat shock proteins are known to modulate polymerization/depolymerization of F-actin and to be involved in cell contraction. Thus, p38 MAPK-mediated phosphorylation of small heat shock proteins provides a potential mechanism of regulation of mesangial cell contractility (6). A schematic representation of the proposed role of Pyk2 in p38 MAPK activation in mesangial cells is depicted in Fig. 13.

It is generally accepted that the mitogenic properties of ET-1 are achieved via activation of the ERK cascade. We have previously shown that biphasic activation of p21\textsuperscript{ras} by ET-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase (9). The rapid and transient initial activation of p21\textsuperscript{ras} is dependent upon the formation of the Shc-Grb2-Sos1 signaling

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**Fig. 9.** Adenovirus-mediated transfer of Pyk2 CRNK inhibits ET-1-induced autophosphorylation of endogenous Pyk2 with different modulating activity upon ERK and p38 MAPK stimulation. Quiescent HMC were infected with either Ad LacZ or Ad Pyk2 CRNK at a m.o.i. of 90 for 24 h, lysates from Ad LacZ- and Ad Pyk2 CRNK-infected cells stimulated with ET-1 for the indicated periods of time (in minutes) were equalized for protein, analyzed by SDS-PAGE, and immunoblotted with phospho-specific anti-Pyk2 anti-Tyr-402 antibody (pY402; first panel). The band shown in the first panel represents the autophosphorylation of endogenous Pyk2, which is inhibited by adenovirus-mediated delivery of Pyk2 CRNK. Cell lysates were also immunoblotted with phosphorylation state-specific anti-ERK antibody (anti-p-Erk; second panel) and phosphorylation state-specific p38 MAPK antibodies (anti-p-p38; fourth panel). To demonstrate equal protein loading, the stripped nitrocellulose was rebotted with anti-ERK antibodies (third panel). B, as assessed by immunofluorescence with anti-FLAG antibodies (Ab), Ad Pyk2 CRNK infected 100% of the cells. Shown is a representative result; the experiment was repeated twice.

**Fig. 10.** Adenovirus-mediated transfer of Pyk2 CRNK does not interfere with ET-1-induced activation of p21\textsuperscript{ras}. Quiescent HMC were infected with either Ad LacZ or Ad Pyk2 CRNK at a m.o.i. of 90 for 24 h and then stimulated with ET-1 for the indicated periods of time (in minutes). GTP-bound active p21\textsuperscript{ras} was isolated by affinity precipitation with a GST-RBD fusion protein, followed by Western analysis with an anti-p21\textsuperscript{ras} antibody (upper panel). The fusion protein was detected by Coomassie Brilliant Blue staining of the polyacrylamide gel (lower panel). The positions of p21\textsuperscript{ras} and GST-RBD are indicated. Some increase in detected p21\textsuperscript{ras} activity at 4 min in cells infected with Ad Pyk2 CRNK and Ad Pyk2 WT, when compared with Ad LacZ-infected cells, can be explained by the difference in the amount of GST-RBD fusion protein used, as shown in the lower panel. The experiment was repeated two times.

**Fig. 11.** Adenovirus-mediated transfer of wild-type Pyk2 and Pyk2 CRNK into RMC does not interfere with ET-1-induced activation of ERK. Lysates from Ad LacZ-, Ad Pyk2 WT-, and Ad Pyk2 CRNK-infected cells, quiescent or stimulated for 4 min with ET-1, were resolved by SDS-PAGE and immunoblotted with anti-Myc (first panel), anti-FLAG (second panel), phosphorylation state-specific anti-ERK (anti-p-Erk; third panel), or anti-ERK (fourth panel) antibodies. WT, wild-type.
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**Fig. 12.** Adenovirus-mediated transfer of Pyk2 CRNK into RMC interferes with ET-1-induced activation of p38 MAPK. Lysates from Ad LacZ- and Ad Pyk2 CRNK-infected cells, quiescent or stimulated for the indicated periods of time (in minutes) with ET-1, were resolved by SDS-PAGE and immunoblotted with phosphorylation state-specific anti-p38 MAPK antibody (anti-p-p38; upper panel). The stripped nitrocellulose was reblotted with anti-P38 MAPK antibodies (lower panel).

**Fig. 13.** Schematic representation of the proposed mechanism by which ET-1 signals through Pyk2 in mesangial cells. ET-1 induces a rapid mobilization of intracellular free calcium and concomitant activation of protein kinase C. Mobilization of free calcium is required for autophosphorylation of Pyk2 on Tyr-402. However, activation of protein kinase C also results in Src-dependent tyrosine phosphorylation of Pyk2. Autophosphorylation of Pyk2 leads to association with Src, resulting in further activation of Src tyrosine kinase activity. Activation of Pyk2 results in the recruitment of unidentified adaptor protein(s) promoting GTP loading of small GTP-binding proteins responsible for activation of the p38 MAPK cascade. PLCβ, phospholipase Cβ; PIP2, phosphatidylinositol bisphosphate; DAG, sn-1,2-diacylglycerol; IP3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; MKK, MAP kinase kinase.

The mechanism of p21ras activation by ET-1 remains uncovered. It is not clear which tyrosine kinase phosphorylates Shc directly and how it is activated in mesangial cells. Persistent Shc phosphorylation is required for the formation of the Shc-Grb2-Sos1 complex, leading to biphase activation of p21ras (9). The fact that activation of p21ras in mesangial cells is dependent upon calcium mobilization argues for the existence of a calcium-dependent event in Shc phosphorylation. It seems unlikely that the autophosphorylation of Tyr-402 in Pyk2, resulting in assembly with Src, is this calcium-dependent event. Our data obtained from the expression of the dominant-negative construct of Pyk2 argue that autophosphorylation of Pyk2 Tyr-402 is not required for p21ras activation in mesangial cells. Since the phosphorylation of Pyk2 Tyr-881 by Src (or other kinases) promotes the direct interaction between Pyk2 and Grb2 (22), the formation of the Pyk2-Grb2-Sos1 signaling complex is possible. However, in ET-1-treated cells, Pyk2 autophosphorylation is rapid and transient; therefore, a putative Pyk2-Grb2-Sos1 complex would exist only very briefly and so is unlikely to be responsible for the second peak of p21ras activation. It is much more possible that some other calcium-dependent event activates c-Src, resulting in rapid activation of Src tyrosine kinase activity. Evidence exists that, in non-neuronal cell types, both Gαi- and Gα11-coupled receptors regulate p21ras in a calcium-dependent manner (50), ultimately leading to MAPK activation. Src family kinases mediate signaling to ERK from both Gαi- and Gα11-coupled receptors (51) and in cells treated with GPCR ligands. Src family kinases represent a point of convergence for signals originating from both focal adhesion complexes and transactivated receptor tyrosine kinases (52). It is of note that, despite the presence of functional epidermal growth factor receptors, ET-1-stimulated epidermal growth factor receptor transactivation was not detected in mesangial cells (data not shown). c-Src is known to be able to phosphorylate Shc, and Shc stays phosphorylated and facilitates the biphase activation of p21ras, leading to the consequent activation of ERK and phosphatidylinositol 3-kinase.

In addition to the interaction between a phosphorylated C-terminal tyrosine residue and their SH2 domains, the closed conformation of Src family kinases is maintained by a second intramolecular association between a proline type II helix region and their SH3 domains (53). It is conceivable that protein kinase C-dependent c-Src activation leads to Pyk2 phosphorylation and a pre-sensitization of this kinase to calcium-dependent activation. In this model, protein kinase C would be responsible for abolishing the SH3-mediated inhibition of c-Src (possibly via conformational changes that would occur subsequent to its phosphorylation of c-Src Ser-12 and Ser-44), and Pyk2 would be responsible for the further opening of this molecule by interfering with the SH2-mediated intramolecular interaction. It is important to note that, in addition to c-Src, other Src family kinases can signal through Pyk2. For example, Pyk2 is selectively phosphorylated by Fyn during T cell antigen receptor signal transduction (54), and we have detected coprecipitation of Pyk2 with both c-Src and Fyn (data not shown). Mesangial cells isolated from mice in which individual Src kinases have been deleted by homologous recombination would undoubtedly prove useful in elucidating the putative role of individual Src kinases in ET-1-mediated Pyk2 signaling.

Under different experimental conditions, Pyk2 has been reported to activate all three major MAPK pathways: ERK (16), JNK (22, 55–57), and p38 MAPK (58). In addition, Pyk2 is critical for the JAK-mediated ERK and STAT1 activation by interferon-γ (59). Since we were not able to detect activation of JNK in glomerular mesangial cells in response to ET-1, we focused our studies on evaluating the role of Pyk2 in signaling via ERK and p38 MAPK. It must be taken into consideration that activation of signaling pathways resulting from overexpression of wild-type Pyk2 could be quite different from those that are mediated by endogenous Pyk2 in response to extracellular stimulus.

The mechanism by which calcium mediates Pyk2 activation is not known. The fact that, in vitro, Pyk2 is directly activated...
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neither by calcium nor by addition of calmodulin suggests that other proteins that mediate calcium-dependent stimulation of Pyk2 exist. Multidomain Pyk2-X-interacting receptor (Nir) proteins are calcium-binding proteins that possess phosphatidylinositol transfer activity and were shown to bind to Pyk2 in vivo and in vitro (60). Of the three Nir proteins thus far described (Nir1, Nir2, and Nir3), only Nir2 is ubiquitously expressed and therefore putatively capable of participating in Pyk2 signaling in mesangial cells. Nir proteins are mammalian homologs of Drosophila RDGβ (retinal degeneration β) protein, and expression of the nir2 gene rescues the phenotype of rdgβ mutant flies (61). It is intriguing that RDGβ has been proposed to participate in the pumping of calcium into intracellular stores (62).

Pyk2 is highly expressed in vascular smooth muscle cells (41), liver epithelial cells (29), cardiac fibroblasts (42), and bone-resorbing osteoclasts (43). In osteoclasts, Pyk2 colocalizes with p130cas and may play a role in the formation of the sealing zone during osteoclast activation (63). Our data, together with findings of other groups, suggest that the role of Pyk2 in coupling GPCRs with activation of tyrosine-dependent signaling pathways is not limited to cells of the nervous system and of hematopoietic origin, but represents a generalized mechanism. Since expression of Pyk2 isoforms generated by alternative RNA splicing has been reported (64, 65), it is possible that cells previously reported to lack Pyk2 express alternative transcripts.

In conclusion, we propose (as summarized in Fig. 13) that p38 MAPK activation in response to ET-1 in mesangial cells occurs via activation of Pyk2 by a mechanism that is dependent on calcium mobilization and stimulation of Src family kinases. As yet, we can only speculate as to how these factors control the “switching on” of Pyk2 signaling. Further studies will be directed toward understanding the fine mechanisms of calcium- and Src kinase-dependent regulation of Pyk2 activity in mesangial cells.

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