Adaptor Proteins Grb2 and Crk Couple Pyk2 with Activation of Specific Mitogen-activated Protein Kinase Cascades*

(Received for publication, December 7, 1998, and in revised form, March 18, 1999)

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The protein tyrosine kinase Pyk2 acts as an upstream regulator of mitogen-activated protein (MAP) kinase cascades in response to numerous extracellular signals. The precise molecular mechanisms by which Pyk2 activates distinct MAP kinase pathways are not yet fully understood. In this report, we provide evidence that the protein tyrosine kinase Src and adaptor proteins Grb2, Crk, and p130Cas act as downstream mediators of Pyk2 leading to the activation of extracellular signal-regulated kinase (ERK) and c-Jun amino-terminal kinase (JNK). Pyk2-induced activation of Src is necessary for phosphorylation of Shc and p130Cas and their association with Grb2 and Crk, respectively, and for the activation of ERK and JNK cascades. Expression of a Grb2 mutant with a deletion of the amino-terminal Src homology 3 domain or the carboxy-terminal tail of Sos strongly reduced Pyk2-induced ERK activation, with no apparent effect on JNK activity. Grb2 with a deleted carboxy-terminal Src homology 3 domain partially blocked Pyk2-induced ERK and JNK pathways, whereas expression of dominant interfering mutants of p130Cas or Crk specifically inhibited JNK but not ERK activation by Pyk2. Taken together, our data reveal specific pathways that couple Pyk2 with MAP kinases: the Grb2/Sos complex connects Pyk2 to the activation of ERK, whereas adaptor proteins p130Cas and Crk link Pyk2 with the JNK pathway.

The MAP kinase family comprises three distinct kinases: extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAP kinase (1). MAP kinases have been implicated in the regulation of several fundamental cellular processes by transmitting extracellular signals from the cell membrane to the nucleus (1–3). Different MAP kinases are activated by signaling pathways composed of small GTPases and cytoplasmic kinase cascades (1). Key components of the ERK pathway include the small GTPase Ras, the serine/threonine kinase Raf, and MAP kinase kinase (Mek), which phosphorylate and activate ERK (4). The JNK pathway is composed of the Rho-related GTPases Rac and Cdc42 and a cytoplasmic kinase cascade in which Mek4 phosphorylates and activates JNK (5, 6). Activated ERK and JNK phosphorylate transcription factors in the nucleus, leading to the modulation of gene expression (7).

In most cases, the rate-limiting step in activation of the ERK and JNK pathways is the conversion of small GTPases from the inactive GDP-bound state to their active GTP-bound form (8, 9). The GDP/GTP exchange is modulated by guanine nucleotide exchange factors (GEFs), which promote formation of the GTP-bound form, and by GTPase activating proteins (GAPs), which stimulate the rate of intrinsic GTP hydrolysis of G-proteins (8). Many GEFs for Ras and Rho-like GTPases identified in mammalian cells are bound to adaptor proteins, such as Grb2, Crk, or Nck (4, 8–10). These adaptor proteins are composed of a SH2 domain and of one or more SH3 domains (10). Upon cell stimulation, the SH2 domains of Grb2, Crk, and Nck bind to tyrosine-phosphorylated docking proteins, such as Shc, IRS-1, Frs2, Gab-1, or p130Cas, or directly to protein receptor tyrosine kinases (4, 10). Thereby, the adaptor protein/GEF complex is translocated to the plasma membrane, where GEFs catalyze the GDP/GTP exchange and activate membrane-bound GTPases (4, 10).

The proline-rich tyrosine kinase (Pyk2) and focal adhesion kinase (FAK) constitute a distinct family of nonreceptor protein tyrosine kinases that are regulated by a variety of extracellular stimuli (11). Pyk2 is predominantly expressed in the central nervous system and cells derived from hematopoietic lineages, whereas its alternatively spliced isoform (Pyk2-H) is specifically expressed in T and B lymphocytes, monocytes, and natural killer cells (12, 13). Pyk2 was implicated in signaling by G protein-coupled receptors, nicotinic acetylcholine receptors, stress stimuli, and membrane depolarization in neuronal cells (12, 14–16). In hematopoietic cells, Pyk2 and Pyk2-H are activated by the inflammatory cytokine tumor necrosis factor α, stimulation of T and B lymphocyte antigen, integrin, interleukin-2, FcRl, and chemokine receptors (13, 16–26). Interactions of Pyk2 with Src family kinases, the Grb2/Sos complex, p130Cas, paxillin, Hic-5, and Graf were reported to regulate intracellular signaling as well as cytoskeletal and morphological changes of cells (12, 14, 18–20, 27–29). In addition, several reports have shown that activation of Pyk2 is necessary for the...
activation of ERK and/or JNK in different cell lines and in response to diverse stimuli (12, 14, 16, 30–33). For example, Pyk2 is required for activation of the JNK pathway but not for ERK in response to angiotensin II or chemokine receptors (31, 33). In PC12 cells, Pyk2 appears to link bradykinin and lysophosphatidic acid receptors with ERK (12, 14) and stress stimuli with the activation of JNK (16). However, the molecular mechanisms by which Pyk2 transmits extracellular signals to specific MAP kinase signaling networks that control diverse cell responses are only partially understood. In this report, we show that Src acts in concert with Grb2/Sos and p130Cas/Crk complexes to mediate Pyk2-induced activation of specific MAP kinase cascades.

**Experimental Procedures**

Reagents—All tissue culture media and antibodies were obtained from Life Technologies and Sigma. LipoFectAMINE was purchased from Life Technologies, Poly(Glu-Tyr) 4:1 and all other chemicals were from Sigma. Aprotinin, leupeptin, and BM chemiluminescence blotting substrate (POD) were obtained from Roche Molecular Biochemicals. Pefabloc SC was obtained from Fluka. Rainbow protein marker, horseradish peroxidase-coupled anti-mouse IgG and [32P]ATP were from American Radiolabeled Chemicals. Poly(Glu-Tyr) was obtained from C. Davis (New York University) and rabbit polyclonal antibodies for hemagglutinin antigen (HA) epitope-tagged ERK2 and JNK1 were kindly provided by C. Marshall (Institute of Cancer Research, London, United Kingdom) and M. Karin, (University of California, San Diego, CA), respectively. Expression vector containing Src kinase inactive mutant (pStGtSrcK) was kindly provided by S. Courtneidge (SUGEN, Inc.). The carboxyl-terminal tail containing Src kinase inactive mutant (pSGTcSrcK) was kindly provided by T. Kirkegaard & Perry Laboratories. Nicktide phosvitin transfer membrane were from Micron Separations, and protein A-Sepharose 4B was from Zymed Laboratories Inc.

**Tissue Culture and Transfections**—Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 µg/ml). Expression vectors for hemagglutinin antigen (HA) epitope-tagged ERK2 and JNK1 were kindly provided by C. Marshall (Institute of Cancer Research, London, United Kingdom) and M. Karin, (University of California, San Diego, CA), respectively. Expression vector containing Src kinase inactive mutant (pStGtSrcK–) was kindly provided by S. Courtneidge (SUGEN, Inc.). The carboxyl-terminal tail containing Src kinase inactive mutant (pSGTcSrcK) was kindly provided by T. Kirkegaard & Perry Laboratories. Nicktide phosvitin transfer membrane were from Micron Separations, and protein A-Sepharose 4B was from Zymed Laboratories Inc.

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anti-HA Western blotting of immune complexes, and the level of GST-c-Jun (1–79) substrate in each lane was visualized by Coomassie Blue staining.

RESULTS

Characterization of Pyk2 Mutants—To investigate the signaling pathways responsible for Pyk2-induced activation of MAP kinases, we generated a series of expression vectors encoding for Pyk2 mutants (Fig. 1A). We first compared activities of Pyk2, Pyk2-Y402F (tyrosine 402, the major autophosphorylation site and direct binding site for the Src SH2 domain, was mutated to phenylalanine), Pyk2-Y881F (tyrosine 881, a putative Grb2 binding site, was changed to phenylalanine), double mutant Pyk2-Y402F/Y881F and PKM to induce Src activation and tyrosine phosphorylation of proteins in human embryonic kidney 293T cells. Expression of Pyk2 or Pyk2-Y881F increased the phosphotyrosine content of cellular proteins and strongly activated endogenous Src proteins, whereas Pyk2-Y402F and Pyk2-Y402F/Y881F were severely impaired (to more than 90%) in their ability to undergo autophosphorylation, activate Src, and phosphorylate other cellular proteins (Fig. 1, B and C). PKM was completely unable to activate Src or to induce any tyrosine phosphorylation of cellular proteins (Fig. 1, B and C).

In order to further analyze the enzymatic properties of different Pyk2 mutants, we compared their in vitro kinase activities, measured by phosphorylation of the substrate poly(Glu-Tyr). Pyk2 and Pyk2-Y881F exhibited similar catalytic activities, whereas Pyk2-Y402F and Pyk2-Y402F/Y881F had decreased activities (15–35%) as compared with the wild type enzyme (Fig. 1D). The observed decrease in catalytic activities in vitro of these mutants could not account for the major reduction in their ability to phosphorylate cellular proteins in vivo (compare Fig. 1D to Fig. 1B). The fact that Pyk2-Y402F and Pyk2-Y402F/Y881F are unable to activate Src (Fig. 1C) indicates that activation of Src kinases by binding to Tyr-402 of Pyk2 plays a critical role in mediating Pyk2-induced phosphorylation of cellular proteins.

Tyrosines 881 and 402 of Pyk2 Are Necessary to Link Pyk2 with Grb2-mediated Pathways—We were further interested to identify cellular proteins that link Pyk2 with the activation of MAP kinases. Pyk2 was suggested to activate ERK by directly binding the Grb2/Sos complex or indirectly via Grb2 binding to tyrosine-phosphorylated Shc proteins (12, 14, 39). We therefore analyzed the ability of different Pyk2 mutants expressed in 293T cells to interact with Grb2. Expression of wild type Pyk2 or Pyk2-Y881F mutant induced phosphorylation of Shc and its association with Grb2 (Fig. 2A), whereas expression of Pyk2-Y402F, Pyk2-Y402F/Y881F, or PKM did not lead to any significant increase in tyrosine phosphorylation of Shc or its association with Grb2 (Fig. 2A). In parallel, the same cell lysates were subjected to immunoprecipitation with anti-Pyk2 and anti-Grb2 antibodies and analyzed by immunoblotting with respective antibodies. Wild type Pyk2 was associated with Grb2, whereas mutation of Tyr-881 to phenylalanine in Pyk2 led to a complete loss of its ability to co-precipitate with Grb2 (Fig. 2B), confirming that Tyr-881 of Pyk2 serves as a direct binding site for Grb2. We also found that Pyk2-Y402F, Pyk2-Y402F/Y881F, and PKM were not able to bind and co-precipitate Grb2 (Fig. 2B). In addition, when Pyk2 was co-transfected with the increasing amounts of a Src kinase inactive mutant (SrcK–), the ability of Pyk2 to bind Grb2 was significantly reduced (Fig. 2C). These data, together with previous findings (12, 39), suggest that the kinase activity of Pyk2, autophosphorylation on Tyr-402 and transphosphorylation of Tyr-881 of Pyk2 by Src kinases are necessary to link Pyk2 with Grb2.

Src Binding to Tyr-402 Mediates Pyk2-induced Activation of ERK and JNK—Because expression of Pyk2 has been shown to

Fig. 1. Characterization of Pyk2 mutants. A, schematic diagram of different Pyk2 forms used in this study. Shading denotes the catalytic region of Pyk2. All constructs were subcloned in the pRK5 expression vector. B, human 293T cells were transfected with expression vectors encoding Pyk2 constructs. Approximately 20 μg of total cell lysates were separated on 7% SDS-PAGE gels and subsequently analyzed by immunoblotting with antibodies against Src and immunoblotted with anti-phosphotyrosine antibodies (anti-PY), and anti-Pyk2 (623). C, the same lysates as in B were subjected to immunoprecipitation with antibodies against Src and immunoblotted with anti-PY and anti-Src (Ab1) antibodies. Src kinase activity was quantified as an increase in phosphorylation of Tyr-416 of Src and presented as mean ± S.D. from three independent experiments. D, Pyk2 or different Pyk2 mutants were immunoprecipitated from lysates of 293T cells and subjected to in vitro kinase reactions using poly(Glu-Tyr) as an exogenous substrate. Fold increase in phosphorylation of poly(Glu-Tyr) is indicated. Bottom panel, a proportion of each Pyk2 immunoprecipitate was separated by 7% SDS-PAGE and immunoblotted with anti-Pyk2 antibodies (623). Results shown are from a representative experiment out of three with similar results.
stimulate both ERK and JNK activities in 293T cells (12, 14, 16), we were further interested to analyze the ability of the Pyk2 mutants to activate different MAP kinases in these cells. Lysates of transfected 293T cells were assayed for activation of ERK2 by immunoblotting with antibodies specific for activated ERK2 or by in vitro kinase reaction using MBP as a substrate. In both experiments, expression of Pyk2 led to approximately 4–5-fold increase in HA-ERK2 activity (Fig. 3A). Expression of Pyk2-Y402F or Pyk2-Y402F/Y881F did not lead to any significant activation of HA-ERK2 kinases (Fig. 3A), whereas the mutation of Tyr-881 in Pyk2 led to approximately 20% reduction in its ability to activate ERK2 when compared with wild type Pyk2 (Fig. 3A). Given the fact that phosphorylation of Shc was intact upon expression of Pyk2-Y881F in the same cells (Fig. 2A), these results suggested that Shc-linked Grb2/Sos pathways may be largely sufficient to substitute for the loss of direct Grb2 binding to Pyk2. Identical results were obtained in 293T cells transfected with Pyk2 and Pyk2 mutants when analyzed for activation of endogenous ERKs (Fig. 3B). PKM was completely unable to activate ERK when transfected in 293T cells (Fig. 3B), which is consistent with previous reports.
(12, 14). In addition, we have previously reported that inhibition of endogenous Src by expression of Csk leads to inhibition of Pyk2-induced ERK activation (14).

We next tested the activation of JNK in the same cells. Expression of wild type Pyk2 in 293T cells led to a 5-fold increase in JNK activity, measured by in vitro phosphorylation of a GST-c-Jun (1–79) fusion protein (16) (Fig. 3C). JNK activation induced by the expression of Pyk2-Y402F, Pyk2-Y402F/Y881F, and PKM was significantly weaker (up to 50–60%) when compared with values obtained by expression of Pyk2 or Pyk2-Y881F (Fig. 3C). In order to strengthen the importance of Tyr-402-mediated Src activation for the ability of Pyk2 to induce JNK activation, we have co-expressed Pyk2 with a Src kinase inactive mutant and analyzed the activation of JNK in these cells. Pyk2-induced increase in JNK activation was significantly reduced in the presence of increasing amounts of a dominant interfering form of Src (Fig. 3D). Together, these findings indicate that the kinase activity of Pyk2 and Tyr-402-linked activation of Src kinases play major roles in pathways connecting Pyk2 with activation of ERK and JNK.

Differential Quantitative Requirements for Grb2 SH3 Domain-mediated Pathways in Regulating Pyk2-induced Activation of ERK and JNK—In order to analyze the involvement of Grb2 and Sos in the Pyk2-induced activation of ERK or JNK, we made use of dominant interfering forms of Grb2 and Sos. Expression of Grb2 variants with a deletion of the carboxy-terminal SH3 domain (Grb2-ΔSH3), a deletion of the carboxy-terminal SH3 domain of Grb2 (Grb2-ΔCSH3), or expression of the carboxy-terminal tail of Sos (Sos-CT) were previously shown to dissociate the endogenous Grb2/Sos complex and act as dominant interfering mutants on activation of the ERK pathway by several stimuli (14, 40, 41). Because Pyk2 can activate ERK by both direct binding of Grb2/Sos complex to Pyk2 or indirectly by binding to Shc (Fig. 2), expression of dominant interfering mutants of Grb2 or Sos should block both pathways by which Pyk2 potentially activates ERK. Expression of Pyk2 together with Grb2-ΔSH3 or Sos-CT led to a strong inhibition of Pyk2-induced ERK activation (Fig. 4A), whereas Grb2-ΔCSH3 was only partially efficient (Fig. 4A). Surprisingly, Grb2-ΔCSH3, but neither Grb2-ΔSH3 nor Sos-CT, reduced the JNK activity upon Pyk2 overexpression (Fig. 4B). We further tested whether Grb2 and CrkII can enhance the ability of Pyk2 to activate MAP kinase pathways. Overexpression of Grb2 to approximately 5-fold over endogenous levels together with Pyk2 led to a strong increase in Pyk2-induced ERK activation, whereas 5-fold overexpression of CrkII did not significantly affect the ability of Pyk2 to activate ERK (Fig. 4A). In addition, co-expression of wild type Grb2 or CrkII with Pyk2 in 293T cells led to an increase in JNK activation as compared with the JNK activation induced by expression of Pyk2 alone (Fig. 4B). It appears, therefore, that a Grb2/Sos complex formation is essential in coupling Pyk2 with ERK, whereas CrkII-mediated and additional Grb2-SH3 domain-mediated pathway may be important for the activation of JNK.

Pyk2 Induces Phosphorylation of p130Cas and Its Association with Crk via Activation of Src Kinases—Because we have observed that co-expression of Pyk2 and the adaptor protein CrkII leads to an increase in JNK activation as compared with cells expressing Pyk2 alone (Fig. 4B), and because it has been demonstrated that the docking protein p130Cas constitutively associates with Pyk2 (18), we were interested to study the role of Crk and p130Cas in Pyk2 signaling. It is known that phosphorylated p130Cas serves as an anchoring protein for the SH2 domain of Crk (42). We therefore examined whether phosphorylated p130Cas recruits Crk upon expression of Pyk2 and tested the requirement of specific tyrosine residues of Pyk2 to induce p130Cas phosphorylation and association with Crk. Expression of Pyk2 or Pyk2-Y881F led to a pronounced phosphorylation of p130Cas and its association with Crk (Fig. 5). In contrast, Pyk2-Y402, Pyk2-Y402F/Y881F, and PKM were unable to induce either tyrosine phosphorylation of p130Cas or its association with Crk (Fig. 5). This also indicates that p130Cas might be phosphorylated by Src, which is activated upon binding to Tyr-402 of Pyk2 (Fig. 1C). Indeed, overexpression of a Src

**Fig. 4.** The Grb2/Sos complex links Pyk2 with ERK activation. A, 293T cells were transiently transfected with HA-ERK2 (0.5 μg) and additional expression vectors: empty vector alone (pRK5), a Pyk2 construct (Pyk2), or Pyk2 together with vectors encoding Grb2, CrkII, Grb2 with a deleted amino-terminal SH3 domain (Grb2-ΔSH3), Grb2 with a deleted SH3 domain (Grb2-ΔCSH3), or the carboxy-terminal tail of Sos (Sos-CT), respectively. ERK2 was immunoprecipitated, and activity was measured by in vitro kinase reactions. The graph indicates percentage in ERK activity relative to that measured with Pyk2 expression vector alone from three independent experiments. Statistical significance was calculated using a Student’s t test for paired samples; *, p < 0.05. B, cells were transfected with HA-tagged JNK1 (0.5 μg) and additional expression vectors: empty vector alone (pRK5), Pyk2 (0.5 μg of Pyk2 + 0.5 μg of pRK5), Pyk2 (0.5 μg) plus 0.5 μg of the following cDNAs: Grb2-ΔSH3, Grb2-ΔCSH3, Sos-CT, Grb2, or CrkII, respectively. JNK kinase assays were performed, and data are presented as differences in JNK activity relative to the values obtained in cells transfected with Pyk2 expression vector alone.
kinase inactive mutant together with Pyk2 led to a decrease in Pyk2-induced p130Cas phosphorylation (Fig. 5A, right panel). The fact that p130Cas constitutively binds to all Pyk2 mutants but recruits Crk only in cells expressing wild type Pyk2 or Pyk2-Y881F suggests that differential phosphorylation of p130Cas regulates signal transmission to Crk proteins (Fig. 5B).

The p130Cas/Crk Complex Specifically Links Pyk2 with the JNK Pathway—We next analyzed a role for p130Cas and Crk in mediating ERK and JNK activities downstream of Pyk2. Deletion of the substrate domain (amino acids 213–514) of p130Cas (p130Cas-DSD) or a mutation in the SH2 domain of Crk (Crk-SH2M-R38V) were previously shown to interfere with endogenous p130Cas/Crk signaling (34, 35). Activation of Pyk2-induced JNK and ERK was analyzed in cells co-expressing Pyk2 with p130Cas-DSD or Crk-SH2M. The Pyk2-induced activation of ERK was not blocked by the presence of p130Cas-DSD or Crk-SH2M (Fig. 6A). In contrast, expression of p130Cas-DSD and Crk-SH2M together with Pyk2 led to strong reduction in the JNK activity when compared with cells expressing Pyk2 alone (Fig. 6B). Hence, the p130Cas/Crk complex seems to specifically link Pyk2 with activation of JNK. These results are consistent with data showing that expression of Crk leads to an increase in JNK, but not a significant increase in ERK activity (Fig. 4) (34).

Recent evidence also suggested that Crk binding to paxillin might play an important role in signaling by IGF-1 and growth hormone receptors (43, 44). Paxillin contains three tyrosines...
(Tyr-31, Tyr-118, and Tyr-181) in Tyr-X-X-Pro motifs that are optimal for binding to the Crk-SH2 domain (45, 46). In addition, it is well established that paxillin constitutively associates with Pyk2 (20, 27) and could thus link Pyk2 to the Crk-mediated activation of JNK. In order to test this hypothesis, we co-expressed Pyk2 with either paxillin or a paxillin triple mutant in which Tyr-31, Tyr-118, and Tyr-187 were mutated to phenylalanine. This mutant form of paxillin is unable to bind to Crk (46). Overexpression of paxillin or paxillin Y31F/Y118F/Y187F mutant did not significantly change the ability of Pyk2 to induce JNK activation, suggesting that Crk binding to paxillin does not link Pyk2 with the JNK pathway in 293T cells (Fig. 6C).

**DISCUSSION**

Pyk2 plays important roles in signal transmission from a broad range of transmembrane receptors toward the MAP kinase module in various cell types (12–33). The expression of dominant interfering mutants of Pyk2 has been shown to block ERK activation in response to membrane depolarization, stimulation of bradykinin, lysophosphatidic acid, or adrenergic G-protein-coupled receptors (12, 14, 32). Pyk2 mutants were also shown to reduce the JNK signaling in response to osmotic stress stimuli, UV irradiation or chemokine treatment (16, 33). So far, very little is known about the signals downstream of Pyk2 that mediate the selective activation of the ERK pathway versus JNK pathway.

In the present study, we have identified Src, Grb2/Sos, and p130Cas/Crk complexes as critical factors in coupling Pyk2 with the activation of MAP kinase cascades (Fig. 7). A mutant form of Pyk2 (Pyk2-Y402F) that is unable to bind and activate Src is impaired in its ability to induce association of Grb2 with Shc, binding of Crk to p130Cas and stimulation of the ERK and JNK cascades. This is supported with data showing that inhibition of Src kinases by expression of Csk or Src kinase inactive mutants not only leads to strong reduction of Pyk2 tyrosine phosphorylation and its association with Grb2, but also inhibits phosphorylation of p130Cas and activation of ERK and JNK induced by Pyk2 (Figs. 1–3 and 5) (14). The functional significance and mechanisms behind the formation of a Pyk2/Src complex resemble those described for interactions between FAK and Src (47, 48). Calalb et al. (49) have demonstrated that phosphorylation of tyrosines 576 and 577 of FAK by Src is necessary for maximal FAK kinase activity. These residues correspond to tyrosines 579 and 580 of human Pyk2 and are located within the activation loop of the catalytic domain, a region responsible for phosphorylation-dependent regulation of protein kinase activity. The observation that Pyk2-Y402F displays partially reduced kinase activity might also indicate that phosphorylation in the catalytic domain of Pyk2 by activated Src is a prerequisite for full kinase activity of Pyk2. It appears therefore that the formation of a Pyk2-Src complex via binding to Tyr-402 might have a dual function. On one hand, it allows Src to phosphorylate Pyk2 within the carboxyl terminus (Tyr-881) and probably within the catalytic domain (Tyr-579 and Tyr-580), which promotes Grb2 binding and enhances Pyk2 kinase activity, respectively. On the other hand, activated Src bound to Pyk2 might directly phosphorylate adjacent cellular proteins, such as Shc and p130Cas, and thus amplifies signals from Pyk2 to downstream effectors. Therefore, it is not surprising that Pyk2-Y402F, which fails to complex with Src, is very weakly tyrosine-phosphorylated and is unable to induce phosphorylation of other cellular proteins (Fig. 1).

It is well established that a key event in regulation of the ERK pathway is the translocation of an adaptor protein/Ras GEF complex, such as Grb2/Sos, to the vicinity of membrane-localized Ras (4). In the case of Pyk2, translocation of the Grb2/Sos complex is mediated by direct binding of the SH2 domain of Grb2 to Tyr-881 of Pyk2 or by association with the tyrosine-phosphorylated adaptor protein Shc (Fig. 2). Expression of Grb2 with a deletion of the amino-terminal SH3 domain or the carboxyl-terminal tail of Sos led to strong inhibition of Pyk2-induced ERK, but no JNK activation (Fig. 4). Hence, the

![Fig. 7. Links between Pyk2 and MAP kinases.](http://www.jbc.org) Pyk2 is activated by various transmembrane receptors via an increase in intracellular Ca<sup>2+</sup> and the activation of PKC. Upon activation, Pyk2 transiently associates with Src by binding of the SH2 domain of Src to autophosphorylated Tyr-402 of Pyk2. This leads to the activation of Src, which in turn phosphorylates Pyk2 and adjacent Shc and p130Cas, thus creating direct binding sites for SH2 domains of adaptor proteins Grb2 and Crk, respectively. Crk can recruit additional proteins to the Pyk2 complex, such as C3G and DOCK180, leading to JNK activation, whereas Grb2 translocates the guanine exchange factor Sos necessary for Pyk2-induced ERK activation.
Grb2/Sos complex is largely necessary for stimulation of the ERK pathway upon activation of Pyk2. These results are consistent with data showing that Grb2 and Sos act downstream of Pyk2 and link lysophosphatidic acid and bradykinin receptors with the ERK cascade (14). Surprisingly, expression of Grb2 with a deletion of the carboxyl-terminal SH3 domain also inhibited Pyk2-induced JNK activity (Fig. 3). This suggests the existence of additional effectors bound to the SH3 domain of Grb2 that are involved in Pyk2-induced activation of JNK. It has been previously observed that the C-SH3 domain of Grb2 binds only weakly to Sos proline-rich sequences and is dispensable for signaling to the Ras pathway in Drosophila (50–52). Furthermore, several biochemical evidence indicates that Grb2 SH3 domains have additional effectors and that multiple pools of Grb2 may exist in mammalian cells. For example, Vav was found to associate primarily with the carboxyl terminus, whereas C3G bound selectively to the amino-terminal SH3 domain of Grb2 (53). Vav contains a Dbl domain able to regulate Rho-like GTPases and thus acts as an upstream regulator of the JNK pathway (54). In addition, the carboxyl-terminal SH3 domain of Grb2 binds strongly to proline-rich sequences of the hematopoietic progenitor kinase-1 (HPK1) (55). HPK1 is a serine/threonine protein kinase that has been shown to interact with the mixed lineage kinase (MLK3), which in turn, can stimulate MEKK4, thereby leading to the activation of JNK (5, 56). Recent findings by Pomerance et al. (57) indicate that the carboxyl-terminal SH3 domain of Grb2 can also bind directly to MEKK1 and thus links EGF receptors with JNK activation. These findings suggest that Grb2 may couple to cytosplasmic serine/threonine kinases via its carboxyl-terminal SH3 domain and directly activate JNK.

Furthermore, our studies have revealed a new role for the p130Cas/Crk complex in signaling by Pyk2. Recent data have shown that Pyk2 expression and activation can enhance the tyrosine phosphorylation of p130Cas, which is constitutively bound to Pyk2 (27). In this report, we show that Src, and not Pyk2 itself, mediates phosphorylation of p130Cas and that formation of a p130Cas/Crk complex specifically links Pyk2 with the activation of JNK (Figs. 5 and 6). Once phosphorylated, p130Cas acts as a docking protein to recruit Crk and its effectors (58, 59). The SH3 domains of Crk bind to several effectors able to activate JNK, including C3G, DOCK180, and Sos (60–62). Pioneering work by H. Hanafusa and co-workers (61) showed that transient expression of Crk leads to the activation of JNK in 293T cells. The fact that dominant interfering mutants of p130Cas and Crk partially inhibited (up to 50–60%) Pyk2-induced JNK activation (Fig. 6), indicated to the presence of redundant signaling pathways involved in Pyk2-mediated JNK activation. Other cellular proteins bound to Pyk2 might therefore be involved in regulation of the JNK cascade. Paxillin is an adaptor protein that constitutively binds to the carboxyl-terminal tail of Pyk2 (20, 27) and is complexed with Crk in IGF-1 and growth hormone-stimulated cells (43, 44). These data indicate that paxillin could couple Pyk2 with Crk-mediated JNK activation. Expression of a paxillin mutant that cannot bind to Crk together with Pyk2 had no effect on the ability of Pyk2 to activate JNK (Fig. 6C), indicating that Crk binding to paxillin is dispensable for Pyk2-mediated JNK activation. In accordance with these data, it has been recently shown that the CrkII/p130Cas, but not CrkII/paxillin, complex formation is required for cytoskeleton organization and anchoragedependent DNA synthesis in Rat-1 fibroblasts (65). Additional cellular proteins, such as Graf (the GTPase-activating protein for Rho), Nirs (aminoterminal domain-interacting receptors), and Pap proteins, were also shown to constitutively associate with Pyk2 and participate in broad range of signaling pathways in cells (28, 66–68). Whether they directly or indirectly participate in Pyk2-induced JNK activation remains to be investigated.

In conclusion, these data indicate that Src acts in concert with adaptor proteins Grb2 and p130Cas/Crk linking Pyk2 to ERK and JNK pathways, respectively. The results of the present study fully support a model in which the formation of Pyk2-Src complexes, by Src binding to autophosphorylated Tyr-402, is critical for phosphorylation of Sos, p130Cas, and Pyk2 itself (Fig. 7). Thereby, binding sites for SH2 domains of the adaptor proteins Grb2 and Crk are created, which in turn recruit different effector proteins that activate ERK and JNK cascades. The specificity in signaling by various Grb2 and Crk effector pathways in different physiological processes remains to be further investigated. One could anticipate that tissue-specific expression and/or mutual interactions of Grb2 and Crk effectors will correlate well with the threshold activation of a target pathway, ERK or JNK, in a particular cell type and in response to specific stimuli.

Acknowledgments—We thank S. Courtneidge, C. Marshall, M. Karin, F. Giancotti, L. Rönström, M. Gischitzky, A. Laudano, M. Matsuda, and H. Hirai for providing reagents. We also thank J. Schlessinger for the initial support in the course of these studies.

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Adaptor Proteins Grb2 and Crk Couple Pyk2 with Activation of Specific Mitogen-activated Protein Kinase Cascades
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doi: 10.1074/jbc.274.21.14893

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