Platelet-derived Growth Factor Requires Epidermal Growth Factor Receptor to Activate p21-activated Kinase Family Kinases*

Received for publication, May 7, 2001
Published, JBC Papers in Press, May 16, 2001
DOI 10.1074/jbc.C100229200

Hong He‡‡, Alexander Levitzki, Hong-Jian Zhu‡‡, Francesca Walker‡, Antony Burgess‡, and Hiroshi Maruta‡‡

From the Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Parkville, Melbourne, Australia 3050 and the Department of Biological Chemistry, Alexander Silvermann Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91090, Israel

The platelet-derived growth factor (PDGF) receptor (PDGFR) transactivates the epidermal growth factor (EGF) receptor (ErbB1) to stimulate the cell migration of fibroblasts through an unknown mechanism (Li, J., Kim, Y. N. & Bertics, P. (2000) J. Biol. Chem. 275, 2951–2958). In this paper we provide evidence that the transactivation of the EGF receptor (EGFR) by PDGFR is essential for PDGF to activate p21-activated kinase (PAK) family kinases. Fetal calf serum (10%) transiently stimulates the PAK activity in NIH 3T3 fibroblasts. The activation of PAK was completely inhibited by either PDGFR-specific inhibitor (AG1295) or EGFR-specific inhibitor (AG1478), suggesting that serum requires either the PDGF- or EGF-dependent pathway or the combination of both to activate PAK. PDGFR-induced activation of PAK is completely inhibited by either AG1295 or AG1478, indicating that PDGFR requires both PDGFR and EGFR for PAK activation. In support of this notion, a mouse embryo fibroblast cell line derived from the EGFR−/− mouse (from Dr. Erwin Wagner) doesn’t activate PAK in response to PDGF. Expression of human EGFR in this cell line restores the ability of the PDGF to induce PAK activation. Our results indicate that PDGF activates PAK through transactivation of ErbB1.

Several distinct Ser/Thr kinases of the PAK1 family such as PAK1, PAK2, and PAK3 are activated by CDC42/Rac GTPases and the SH3 proteins called PIX/Cool (1). PAKs are required for PAK1, PAK2, and PAK3 are activated by CDC42/Rac GTPases.

This paper is available online at http://www.jbc.org

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The mouse E12.5 embryonic fibroblast cell line (EFES-1) was derived from an ErbB1-deficient mouse (C57BL/6) generated by ErbB1 gene knock-out (14) and is a generous gift from Dr. Erwin Wagner (Research Institute of Molecular Pathology, Vienna, Austria). Both EFES-1 and normal mouse NIH 3T3 fibroblasts (16) were grown in a standard medium, i.e. Dulbecco’s modified Eagle’s medium in the presence or absence of 10% fetal calf serum as described previously (3, 4, 15, 16). The PDGF receptor-specific inhibitor AG1295 and the ErbB1-specific inhibitor AG1478 were synthesized as described previously (10). The following antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA: anti-PAK antibody, anti-EGFR antibody, anti-PDGF receptor antibodies, and anti-phospho-Tyr antibody. Anti-PDGF receptor antibodies were also purchased from PharMingen. Murine EGFR was purified from mouse submaxillary glands in accordance with a published procedure (17). Purified human PDGFR was purchased from Calbiochem.

Transfection of EGFR-deficient Fibroblast Cell Line EFES-1 with Human EGFR—To overexpress human EGFR in the EGFR-null mouse cell line EFES-1, the cells were cotransfected with a pcDNA3 vector (Invitrogen) carrying M2-tagged human EGFR cDNA (18) and a second vector carrying puromycin-resistance selection plasmid (19). The mouse E12.5 embryonic fibroblast cell line (EFES-1) was derived from an ErbB1-deficient mouse (C57BL/6) generated by ErbB1 gene knock-out (14) and is a generous gift from Dr. Erwin Wagner (Research Institute of Molecular Pathology, Vienna, Austria). Both EFES-1 cells and normal mouse NIH 3T3 fibroblasts (16) were grown in a standard medium, i.e. Dulbecco’s modified Eagle’s medium in the presence or absence of 10% fetal calf serum as described previously (3, 4, 15, 16). The PDGF receptor-specific inhibitor AG1295 and the ErbB1-specific inhibitor AG1478 were synthesized as described previously (10). The following antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA: anti-PAK antibody, anti-EGFR antibody, anti-PDGF receptor antibodies, and anti-phospho-Tyr antibody. Anti-PDGF receptor antibodies were also purchased from PharMingen. Murine EGFR was purified from mouse submaxillary glands in accordance with a published procedure (17). Purified human PDGFR was purchased from Calbiochem.

Transfection of EGFR-deficient Fibroblast Cell Line EFES-1 with Human EGFR—To overexpress human EGFR in the EGFR-null mouse cell line EFES-1, the cells were cotransfected with a pcDNA3 vector (Invitrogen) carrying M2-tagged human EGFR cDNA (18) and a second vector carrying puromycin-resistance selection plasmid (19). The mouse E12.5 embryonic fibroblast cell line (EFES-1) was derived from an ErbB1-deficient mouse (C57BL/6) generated by ErbB1 gene knock-out (14) and is a generous gift from Dr. Erwin Wagner (Research Institute of Molecular Pathology, Vienna, Austria). Both EFES-1 cells and normal mouse NIH 3T3 fibroblasts (16) were grown in a standard medium, i.e. Dulbecco’s modified Eagle’s medium in the presence or absence of 10% fetal calf serum as described previously (3, 4, 15, 16). The PDGF receptor-specific inhibitor AG1295 and the ErbB1-specific inhibitor AG1478 were synthesized as described previously (10). The following antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA: anti-PAK antibody, anti-EGFR antibody, anti-PDGF receptor antibodies, and anti-phospho-Tyr antibody. Anti-PDGF receptor antibodies were also purchased from PharMingen. Murine EGFR was purified from mouse submaxillary glands in accordance with a published procedure (17). Purified human PDGFR was purchased from Calbiochem.

ASSAY FOR PAK KINASE ACTIVITY—Serum-starved NIH 3T3, EFES-1, or E12.5 mouse embryonic fibroblast cells were stimulated by 10% serum, EGF (100 ng/ml), or PDGF (10 ng/ml) in the presence or absence of AG1478 (0.2 mM), AG825 (0.8 mM), or AG1295 (1 mM) for specified times. Cells were lysed in the lysis buffer (40 mM HEPES, pH 7.4, 1% Nonidet P-40, 1 mM EDTA, 100 mM NaCl, 25 mM NaF, 100 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride, and 100 units/ml aprotinin). The lysates containing 1 µg of protein (as measured by Bradford assay) were then immunoprecipitated with anti-PAK antibody (Santa Cruz), and the immunoprecipitates were subjected to the PAK kinase assay as described previously (2, 3). Immunoblotting was performed to determine the PAK protein level.

Immunoprecipitation and Immunoblotting—Serum-starved EGFR/EFES-1 cells were stimulated by either EGF (100 ng/ml) or PDGF (50 ng/ml) for 10 min in the presence or absence of either AG1478 (0.2 mM) or AG1295 (1 mM). Cell lysates containing 1.5 µg of protein (as measured by Bradford assay) were precleared with protein A-Sepharose beads (Amersham Pharmacia Biotech) and then incubated with either anti-EGF receptor (Santa Cruz) or anti-PDGF antibodies (Santa Cruz) and protein A-Sepharose beads (13). The proteins in immunoprecipitates were separated on 7.5% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Micron Separations, Inc.). The membrane was blocked with 10% (w/v) skim milk in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) at 4 °C overnight and then incubated for 1 h at room temperature with anti-phospho-Tyr antibody (Santa Cruz). After washing with TBST, the blot was incubated with horseradish peroxidase-conjugated anti-mouse secondary...
antibodies (Bio-Rad). The bound antibodies were visualized using ECL reagents (Amersham Pharmacia Biotech). To determine the amount of the receptors in the immunoprecipitates, the same membranes were stripped with 2% SDS and 100 mM dithiothreitol in 62.5 mM Tris, pH 6.4, for 30 min at 55 °C and washed with TBST. The blots were then reblotted with anti-EGFR or anti-PDGFR (PharMingen) antibodies.

RESULTS

Serum-induced PAK Activation Requires Both PDGF Receptor and ErbB1—Within 10 min, fetal calf serum (10%) transiently activated PAK in serum-starved normal NIH 3T3 fibroblasts (Fig. 1A). Under these conditions, no increase in PAK protein levels is detectable. To identify the signaling pathway(s) involved in serum-induced PAK activation, we examined the effects of the following three specific tyrosine kinase inhibitors: the PDGF receptor inhibitor AG1295 (10), the EGF receptor inhibitor AG1478 (10), and the ErbB2-specific inhibitor AG825 (10). Either AG1295 (1 mM) or AG1478 (200 nM) inhibited completely the serum-induced PAK activation, whereas AG825 (800 nM) only partially inhibited the PAK activation (Fig. 1B). The particular concentrations of each inhibitor were chosen from published data (10), and our experiments demonstrated that the PAK kinase was not inhibited directly by any of these inhibitors (data not shown). These results indicated that serum-induced PAK activation required both PDGF receptor- and EGF receptor-dependent pathways.

PDGF Requires Both PDGF Receptor and ErbB1 for PAK Activation—To clarify the above assumptions, we have examined the effects of PDGF and EGF on PAK activation separately. Like serum, PDGF (10 ng/ml) alone transiently activated PAK (Fig. 2A) as described previously (3, 9). The PDGF receptor inhibitor AG1295 (1 mM) blocked the PDGF-induced PAK activation (Fig. 2B). Surprisingly, the ErbB1-specific in-
hbitor AG1478 also completely inhibited the PDGF-induced PAK activation at the concentration of 200 nM. The results suggest that PDGF requires EGFR, in addition to PDGFR, for PAK activation. Although EGF (100 ng/ml) also transiently stimulates PAK activity (Fig. 3A), the EGF-induced PAK activation was inhibited by AG1478 (Fig. 3B). Clearly PDGF receptor activation is bypassed when EGF stimulates PAK activity. These observations strongly support our first hypothesis that PDGF receptor signaling transactivates EGFR, and they exclude the second hypothesis.

**PDGF Does Not Activate PAK in Embryonic Fibroblasts from EGFR-deficient Mice**—Although the complete inhibition of PDGF-induced PAK activation by the EGFR-specific inhibitor AG1478 (200 nM) strongly supports the notion that PDGF requires EGFR for PAK activation in NIH 3T3 cells, these experiments clearly do not exclude the possibility that this compound at the concentration used inhibits the function of other cellular protein(s), which happen to be essential for both EGFR- and PDGFR-induced PAK activation. Therefore, to explore further the notion that PDGF requires ErbB1 to activate PAK kinase, we used the embryonic fibroblast cell line (EFES–1) derived from ErbB1-deficient (−/−) mice (14). There

**Fig. 3.** **EGF does not require PDGFR to activate PAK.** Serum-starved NIH 3T3 cells were stimulated by EGF (100 ng/ml) for the indicated time (A) or for 10 min in the presence or absence of either AG1478 (0.2 μM) or AG1295 (1 μM) (B). The cell lysates were subjected to PAK kinase assay as described in Fig. 1. The relative PAK kinase activity was calculated as -fold stimulation by EGF of the control PAK kinase activity. The values in the graph are the means ± S.D. obtained from three independent experiments.

**Fig. 4.** **PDGF cannot activate PAK without EGF receptor.** A, EGF receptor is essential for PDGF-induced PAK activation. Serum-starved EFES–1 cells were stimulated by PDGF for the indicated time periods. No significant PAK activation was detected although the PDGFR was activated as shown by receptor tyrosine phosphorylation (top panel). The values in the graph are the means ± S.D. obtained from three independent experiments. B, EGF receptor expression restores PDGF-induced PAK activation. Serum-starved, EGFR/EFES–1 cells were stimulated by PDGF (10 ng/ml) for 10 min in the presence or absence of either AG1478 (0.2 μM) or AG1295 (1 μM). PAK was transiently activated by PDGF in these cells. The activation of PAK was inhibited by both EGFR- and PDGFR-specific inhibitors. Similar levels of PAK protein were detected in each lane (middle panel). The values in the graph summarize data from three independent experiments.
was no PAK activation by PDGF in EFE8–1 cells although PDGF receptor is activated and becomes tyrosine-phosphorylated (Fig. 4A). Expression of human EGFR in this cell line restored the ability of PDGF to activate PAK (Fig. 4B). Again this response is inhibited by both AG1478 and AG1295 (Fig. 4B). Clearly the EGFR receptor is required for PDGF-induced PAK activation.

**PDGF Receptor Transactivates the EGFR—**Although EGFR activates PAK in NIH 3T3 fibroblasts (Fig. 3), the EGFR levels in this cell line are very low (20). Using the method described, we were unable to detect EGFR protein or EGFR-mediated tyrosine phosphorylation of EGFR receptor (data not shown). However, in the EGFR/EF8–1 cell line, PDGF clearly stimulates tyrosine phosphorylation of the EGFR receptor (Fig. 4C). The results indicate that the PDGFR is active in these cells following exposure to PDGF and that PDGFR transactivates EGFR, which is responsible for PAK activation. However, it still remains to be clarified how PDGF receptor activates EGFR.

**DISCUSSION**

In the present study, we have observed that PDGF activates PAK via EGFR receptor, and the EGFR receptor becomes tyrosine-phosphorylated in response to PDGF treatment, suggesting that the transactivation of EGFR by PDGF (11–13) is involved in PDGF-induced activation of PAK. PAK is required for normal cell motility and directed migration (21, 22). In particular PAK is required for PDGF-stimulated cell migration (23, 24). PDGF activates PAK, which then colocalizes with F-actin in lamelipodia and membrane ruffles, suggesting a role of PAK in directed cellular movement by regulating actin dynamics at the leading edge of cells. Recently it has been shown that PDGF-stimulated cell migration depends on the EGFR receptor and that the EGFR receptors become tyrosine-phosphorylated in response to PDGF stimulation (13). This is consistent with our observations in the present study. Why does PDGF rely on the EGFR receptor to induce cell migration? Our present observation suggests that the EGFR receptor is required for PDGF to activate PAK, which regulates actin dynamics and consequentially leads to cell migration.

Like oncogenic RAS mutants, activation of the PDGF receptor directly activates PI3K (25), which in turn activates both CDC42 and Rac, which should lead to PAK activation (26–28). However, this well characterized PI3K pathway alone does not appear to be sufficient for PAK activation. We have demonstrated here that the PDGF receptor requires the EGFR receptor for PAK activation. The oncogenic RAS mutants such as v-Ha-RAS also require the activation of the EGFR receptor (3). Why do both PDGF and oncogenic RAS require EGFR receptor activation? Because both PDGF and EGFR are able to recruit PAK to the plasma membranes through the SH2/SH3 protein NCK, which directly binds the N-terminal Pro-rich motif of PAK and the activated (Tyr-phosphorylated) PDGF and EGFR (9, 25, 26), it is unlikely that PDGF requires EGFR for the NCK-binding per se. Interestingly, the Ras-induced PAK activation absolutely requires a member of Src family kinases (3, 4). Src family kinases tyrosine-phosphorylate the CAT (Coo-associate tyrosine-phosphorylated protein) family proteins, which in turn bind to the SH3 family proteins called PIX/Cool (29). The SH3 family protein PIXs/Cools then bind with the N-terminal Pro-rich motif of PAK and stimulate PAK activity (30). Although both PDGFR and ErbB family kinases are known to activate Src family kinases (3, 4, 31), it still remains to be clarified whether the PDGFR-associated activation of PAK requires an ErbB kinase to activate Src family kinases.

Because the sensitivity of PDGF-induced PAK activation to the three specific tyrosine kinase inhibitors is basically identical to that of the serum-induced one, it is likely that the dominant PAK-activating factors in serum are PDGF family ligands but not EGF family ligands for the following reasons. First, 200 nM AG1478 does not inhibit either the PDGF receptor or ErbB2 as the IC50 of AG1478 for both PDGF receptor and ErbB2 is higher than 100 μM (10). Second, as the IC50 of AG1295 for both EGFR and ErbB2 is higher than 100 μM (10), 1 μM AG1295 does not inhibit ErbB family receptors. Finally, the IC50 of AG825 for EGF receptor and PDGF receptor are 20 and 40 μM, respectively (10), again making it unlikely that 0.8 μM AG825 exerts any significant effect on these non-ErbB receptors.

**Acknowledgment—**We are very grateful to Dr. Erwin Wagner for a generous gift of the mouse ErbB1-deficient (−/−) fibroblast cell line EFE8–1 and Dr. Calle Heldin for critical reading of the manuscript.

**REFERENCES**

1. Manser, D., Loo, Th., Koh, C. G., et al. (1998) Mol. Cell 1, 183–192
2. Obermeier, A., Ahmed, S., Manser, E., et al. (1998) EMBO J. 17, 4328–4339
3. He, H., Hirokawa, Y., Manser, E., Lim, L., Levitzki, A. & Maruta, H. (2001) Cancer J. (Boston) 7, 191–202
4. He, H., Hirokawa, Y., Levitzki, A. & Maruta, H. (2000) Cancer J. Sci. Am. 6, 243–248
5. Glick, A., Sporn, M., & Yuspa, S. (1991) Mol. Caregic. 4, 210–219
6. Higashiyama, S., Abraham, J., Miller, J., et al. (1991) Science 251, 936–939
7. Nermanno, N., Selvan, M., Qi, C., et al. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2790–2794
8. Miccione, G., Bianco, C., Kannan, S., et al. (1996) J. Cell. Biochem. 60, 437–446
9. Bokoch, G., Wang, Y., Bohl, B., et al. (1996) J. Biol. Chem. 271, 25746–25749
10. Levi, Z., & Gazi, A. (1995) Science 267, 1782–1788
11. Collins, M., Sinnott-Smith, J. & Rozengurt, E. (1983) J. Biol. Chem. 258, 11689–11693
12. Walker, F. & Burgess, A. W. (1991) J. Biol. Chem. 266, 2746–2752
13. Li, J., Kim, Y. N. & Bertics, F. (2000) J. Biol. Chem. 275, 2951–2958
14. Sibilia, M. & Wagner, E. (1995) Science 269, 234–238
15. Valgeirsdottir, S., Eriksson, A., Nister, M., Heldin, C.-H., Westmark, B. & Claesson-Welsh, L. (1993) J. Biol. Chem. 268, 10161–10170
16. Maruta, H., Holden, J., Sizeland, A. & D’abaco, G. (1991) J. Biol. Chem. 266, 1661–1668
17. Walker, F. & Burgess, A. W. (1988) Biochem. J. 266, 109–115
18. Ullrich, A., Coussens, L., Hayflick, J., et al. (1984) Nature 309, 418–425
19. Zhu, H. J., Jaria, J. & Sizeland, A. (1999) J. Biol. Chem. 274, 32258–32264
20. Okuyama, M. A., Beurink, I., Hersch, K., Daly, J. M. & Hynes, N. E. (1999) J. Biol. Chem. 274, 17209–17218
21. Kisses, W. B., Daniels, H. R., Otey, C., et al. (1999) J. Cell Biol. 147, 831–843
22. Sells, M. A., Boyd, J. T. & Chernoff, J. (1999) J. Cell Biol. 145, 837–849
23. Dharmawadhane, S., Schurmann, A., Sells, M., et al. (2000) Mol. Cell. Biol. 11, 3341–3352
24. Dharmawadhane, S., Sanders, L. C., Martin, S. S., et al. (1997) J. Cell Biol. 138, 1256–1257
25. Suza, M., Keeler, M. & Varticovski, L. (1992) J. Biol. Chem. 267, 22951–22956
26. Downward, J. (1998) in G Proteins, Cytoskeleton and Cancer (Maruta, H. & Kohama, K., eds) pp. 171–183, Landes Bioscience, Austin, TX
27. Maruta, H., He, H., Tikoo, A., Shen, T. & Nur-E-Kamal, M. S. A. (1999) Microsc. Res. Tech. 47, 61–67
28. Liliental, J., Moon, S. Y., Lesche, R., et al. (2000) Curr. Biol. 10, 401–404
29. Boguski, S., Bailey, D., Lenard, Z., Hart, M., Guan, J. L., Premont, R. T., Taylor, S. J. & Cerione, R. A. (1999) J. Biol. Chem. 274, 22389–22400
30. Daniels, R. H., Zenke, F. T. & Bokoch, G. M. (1999) J. Biol. Chem. 274, 6047–6050
31. Raleigh, R. & Bishop, J. M. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 7845–7849
Platelet-derived Growth Factor Requires Epidermal Growth Factor Receptor to Activate p21-activated Kinase Family Kinases
Hong He, Alexander Levitzki, Hong-Jian Zhu, Francesca Walker, Antony Burgess and Hiroshi Maruta

J. Biol. Chem. 2001, 276:26741-26744.
doi: 10.1074/jbc.C100229200 originally published online May 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.C100229200

 Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 19 of which can be accessed free at
http://www.jbc.org/content/276/29/26741.full.html#ref-list-1