TYK2, a Janus kinase, plays both structural and catalytic roles in type I interferon (IFN) signaling. We recently reported (Rani, M. R. S., Gauzzi, C., Pellegrini, S., Fish, E., Wei, T., and Ransohoff, R. M. (1999) J. Biol. Chem. 274, 1891–1897) that catalytically active TYK2 was necessary for IFN-β to induce the β-R1 gene. We now report IFN-β-mediated activation of STATs and other components in U1 (TYK2-null) cell lines that were complemented with kinase-negative (U1.KR930) or wild-type TYK2 (U1.wt). We found that IFN-β induced phosphorylation on tyrosine of STAT3 in U1.wt cells but not in U1.KR930 cells, whereas STAT1 and STAT2 were activated in both cell lines. Additionally, IFN-β-mediated phosphorylation of interferon-α receptor-1 (IFNAR-1) was defective in IFN-β treated U1.KR930 cells, but evident in U1.wt cells. In U1A-derived cells, the p85/p110 phosphoinositol 3-kinase isoform was associated with IFNAR-1 but not STAT3, and the association was ligand-independent. Further, IFN-β treatment stimulated IFNAR-1-associated phosphoinositol kinase activity equally in either U1.wt or U1.KR930 cells. Our results indicate that catalytically active TYK2 is required for IFN-β-mediated tyrosine phosphorylation of STAT3 and IFNAR-1 in intact cells.

The role(s) of the tyrosine kinase TYK2 in the type I IFN signaling pathway has been demonstrated through studies carried out in the TYK2-minus cell line U1A (1, 2). U1A cells are completely refractory to IFN-α, yet retain a partial responsive-ness to IFN-β (2), suggesting that IFN-β, but not IFN-α, activates both TYK2-dependent and -independent signaling pathways (3). Reconstitution of U1A cells with wild-type or mutant forms of TYK2 has revealed a surprising diversity of structural and catalytic functions for TYK2 in the type I IFN receptor (IFNAR-1/2) complex. For example, the N region (residues 1–591) of TYK2 was required for stable cytoplasmic accumula-

Catalytically Active TYK2 Is Essential for Interferon-β-mediated Phosphorylation of STAT3 and Interferon-α Receptor-1 (IFNAR-1) but Not for Activation of Phosphoinositol 3-Kinase*

(Received for publication, May 25, 1999, and in revised form, September 2, 1999)

M. R. Sandhya Rani‡, Douglas W. Leaman§, Yulong Han‡, Stewart Leung¶, Alan Fish,** and Richard M. Ransohoff††‡‡

From the Departments of ‡Neurosciences, **Cell Biology, and ¶Molecular Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195, †Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario M5S 3E2, Canada, and §Berlex Biosciences, Richmond, California 94804-0099

* This work was supported by grants from the National Institutes of Health (1RO1 CA62220), to R. M. R., the National Multiple Sclerosis Society (RG 2362), Berlex Biosciences, and Williams Family Fund for Multiple Sclerosis Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Dept. of Neurosciences, Lerner Research Institute, NC30, The Cleveland Clinic Foundation, Cleveland, OH 44195. Tel.: 216-444-0627; Fax: 216-444-7927; E-mail: ransohr@ccf.org.

† The abbreviations used are: IFN, interferon; PI3K, phosphatidylinositol 3-kinase; GDAC, genomic DNA affinity chromatography; PVD, polyvinylidene difluoride; IFNAR, interferon-α receptor; PAGE, polyacrylamide gel electrophoresis.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
immunoprecipitation was done at 4 °C for 3 h. The immunoprecipitates were coupled to protein A-agarose beads for 1 h at 4 °C with rotation, and assay. For immunoprecipitation, anti-IFNAR-1 antibodies (22) were used to precipitate tyrosine-phosphorylated STAT3 and IFNAR-1 in U1A-derived cell lines. We found PI3K to co-immunoprecipitate with IFNAR-1, but not STAT3. Furthermore, IFNAR-1-associated PI3K activity was markedly elevated by IFN-β in the presence or absence of catalytically active TYK2, consistent with the observation that PI3K association with the IFNAR-1 receptor component was phosphotyrosine-independent. Thus, it appears that in these cells TYK2 and PI3K activation are not interdependent, suggesting that these molecules have distinct downstream signaling functions.

MATERIALS AND METHODS

Cell Lines and Interferons—Human fibrosarcoma 2TGH cells, mutant U1A, and derivative cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (2). U1.wt and U1.KR930 cells described earlier (10) were maintained in Dulbecco’s modified Eagles medium with 10% calf serum with 250 µg/ml of hygromycin and 450 µg/ml of G418 (5). Purified recombinant IFN-α2 (1 × 10^6 units/ml) was obtained from Wellcome Research Laboratories (Kent, United Kingdom), and recombinant IFN-β-1b (2 × 10^5 units/mg protein) was from Berkeley Biosciences (Richmond, CA). IFNs were used at a final concentration of 1,000 units/ml unless stated otherwise.

Cell Extracts and Genomic DNA Affinity Chromatography (GDAC)—Cells were treated with 15,000 units/ml of IFN for 15 min, and nuclear extracts were prepared as described previously (18). Briefly, cells were washed twice with ice-cold phosphate-buffered saline that contained 1 mM Na2VO4 and 5 mM NaF and once with hypotonic buffer. Cells were lysed in hypotonic buffer containing 0.2% Triton X-100 and nuclear extracts collected by differential centrifugation. GDAC has been previously described (18).

50 µg of nuclear extract was incubated for 20 min with 25 µg of poly(dI-dC) (Amersham Pharmacia Biotech) in binding buffer as described previously (18). This mixture (200 µl) was incubated for 2 h with 100 µl of bovine genomic DNA-cellulose (Sigma). The DNA-binding proteins were eluted in high salt buffer, concentrated, and resolved by SDS-PAGE for analysis in Western immunoblotting experiments (18).

Western Immunoblot—Cells were treated with recombinant IFN-β at 37 °C for 10–15 min before preparation of cell extracts. The antibodies used were: anti-STAT1 and anti-STAT2 (Transduction Laboratories, Lexington, KY), anti-STAT3 (D. Levy, NY University), anti-p85 (Upstate Biotechnology, Inc., Lake Placid, NY). STAT proteins were immunoprecipitated from extracts (19, 20), separated by SDS-polyacrylamide gel electrophoresis, and adsorbed to polyvinylidene difluoride (PVDF) membranes (Stratagene, La Jolla, CA). Incubation with primary antibody was for 2 h at room temperature. Blots were washed thoroughly followed by incubation with secondary antibody for 1 h at room temperature. Immuno-reactive bands were visualized with the ECL Western blotting system (Amersham Pharmacia Biotech). Tyrosine phosphorylation was monitored by Western blotting using anti-phosphotyrosine monoclonal antibodies PY20, (Transduction Laboratories) and 4G10 (Upstate Biotechnology, Inc.).

In Vitro Phosphoinositol Kinase Assay—Cells at 70–80% confluency in 150-mm-diameter culture dishes were serum-starved (0% fetal bovine serum) for 4 h to suppress endogenous PI3K activity (21), and treated with IFN-β for varying times. Cells were lysed with buffer containing 1% Nonidet P-40, 50 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 50 mM NaF, 1 mM Na3VO4, 14 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 10 µg/ml of aprotinin, leupeptin, and pepstatin. Protein concentrations in cell extracts, determined by Bradford reactions, were equalized for each sample. Following immunoprecipitation, anti-IFNAR-1 antibodies (22) were precoupled to protein A-agarose beads for 1 h at 4 °C with rotation, and immunoprecipitated was done at 4 °C for 3 h. The immunoprecipitates were washed with lysis buffer 4 to 5 times. The kinase assay in 50 µl contained 10 µg of phosphatidylinositol (Sigma), 200 µM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 10 mM MgCl2, and 10 µM ATP and [γ-32P]ATP (specific activity 6,000 Ci/mmol, 10 µCi/sample). After incubation for 30 min at 37 °C, an equal volume of water was added, and the reaction was stopped with 300 µl of MeOH-CHCl3 (2:1). After the sample was mixed, 100 µl of water and 200 µl of CHCl3 was added. After vortexing, the organic phase was collected, dried, and resuspended in 25 µl CHCl3-MeOH (1:1) and spotted on thin-layer chromatography plates. The plates were developed in CHCl3-MeOH:4N NH4OH:water (45:30:3:5), dried, and exposed to X-O-mat film (Eastman Kodak). The signal was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

IFN-β-mediated Activation of STAT3 Requires Catalytically Active TYK2—The availability of sibling cell lines expressing equal amounts of catalytically active (U1.wt) or kinase-deficient (U1.KR930) TYK2 (6) provided an opportunity to examine the requirement for catalytic TYK2 in IFN-mediated STAT activation. This question was addressed using GDAC, a technique that monitors activated STATs without selection bias for DNA binding elements (18). U1.wt and U1.KR930 cells generated activated STAT1 and STAT2 upon IFN-α (results not shown) or β treatment (Fig. 1a). GDAC failed to recover STAT3 from lysates of IFN-treated U1.KR930 cells (Fig. 1a). The activation of STAT1 and STAT2 was 30–40% lower in the U1.KR930 cells compared with U1.wt cells as determined by densitometry analyzed using NIH Image analysis v1.65. The dark bars in the graph represent the U1.wt cells and the light bars represent the U1.KR930 cells. The expression of STAT1 and STAT2 in the U1.wt cells was set at 100%.

Phosphorylation of tyrosine on STAT1, -2, and -3 was assayed by immunoprecipitation Western blotting experiments.
STAT1 and STAT2 were activated in U1.wt and U1.KR930 cells in response to IFN-β (Fig. 2a). Consistent with results obtained by GDAC, STAT1 and STAT2 phosphorylation was decreased by 45–50% in the U1.KR930 cells compared with U1.wt cells as determined by densitometry with NIH Image analysis v1.65 (Fig. 2c). No activation of STAT3 was seen in U1.KR930 cells treated with IFN-β (Fig. 2b) although equivalent amounts of STAT3 protein were present in both the cell lines. The absence of activated STAT3 from IFN-induced DNA-binding complexes was confirmed by using electrophoretic mobility shift assays with an oligonucleotide probe for the c-sis-inducible element. The c-sis-inducible element is a growth factor-responsive element, originally identified in the c-fos promoter, that binds homo- and heterodimers of STAT1 and STAT3 (23). No STAT3-containing DNA-binding complexes were detected in the extracts from U1.KR930 cells (results not shown). These results, along with the observations defined by GDAC, indicate that STAT3 activation requires kinase function of TYK2.

Catalytically Active TYK2 Is Essential for IFN-β-mediated Phosphorylation in Intact Cells—Binding of type I IFN to its receptor induces rapid tyrosine phosphorylation of the receptor subunits IFNAR-1 and IFNAR-2. In vitro studies had revealed that TYK2 directly binds and phosphorylates IFNAR-1 (9, 24). STAT3 has also been shown to associate with IFNAR-1 in a tyrosine phosphorylation-dependent manner (11). In contrast, experiments using IFN-treated U266 cells showed no association of STAT3 with IFNAR-1 (25). In that study, tyrosine phosphorylation of STAT3 was proposed to be essential for association with PI3K. In contrast, experiments using IFN-treated U266 cells showed no associa-
IFN-β-stimulated (0% fetal bovine serum) for 4 h, reserved as controls or treated in...regardless of the presence of ligand.

Proteins were resolved by SDS-PAGE, blotted onto PVDF membranes, and probed with anti-p85 antibodies. IFNAR-1 and p85 co-immunoprecipitated regardless of the presence of ligand. Panel a, IFN-β-mediated activation of p85 does not require catalytically active TYK2. Cells were serum-starved (0% fetal bovine serum) for 4 h, reserved as controls or treated with IFN-β-1b (2,500 units/ml) for 10 min before preparation of cell extracts, and immunoprecipitated with antibodies to IFNAR-1. Proteins were resolved by SDS-PAGE, blotted onto PVDF membranes, and probed with anti-p85 antibodies. IFNAR-1 and p85 co-immunoprecipitated regardless of the presence of ligand. Panel b, IFN-β-mediated activation of p85 with Tyk2, Jak1, IFNAR-1, or IFNAR-2c (17). In U1A-derived cells, we found p85 to co-immunoprecipitate with IFNAR-1 in the presence or absence of IFN-β in both U1.wt and U1.KR930 cells (Fig. 4a). IFN-inducible phosphatidylinositol kinase activity was detected in anti-IFNAR-1 immunoprecipitates, regardless of the presence of catalytically active TYK2 (Fig. 4b). A 4–5-fold IFN-inducible increase in lipid kinase activity was observed in both cell lines.

We also examined the role of catalytically active TYK2 in association of STAT3 with p85 in the presence and absence of IFN-β. The p85 regulatory subunit of PI3K failed to co-precipitate with anti-STAT3 (as shown in Fig. 4c), and failed to associate with phosphopeptides derived from STAT3 (results not shown). Further, STAT3 tyrosine phosphorylation did not induce p85 association in U1.wt cells. PI-specific lipid kinase activity was not detected in STAT3 immunoprecipitates by in vitro kinase assay. We concluded that activated STAT3 does not mediate adaptor function for recruitment of p85 to the IFN-α/β receptor in U1A-derived cells.

**DISCUSSION**

We addressed the function of type I IFN receptor signaling components and TYK2 kinase by studying human fibrosarcoma cells that possess all structural elements of the type I IFN receptor but lack the kinase function of TYK2. We found that catalytically active TYK2 is essential for IFNAR-1 phosphorylation by type I IFNs. In vitro studies had indicated that IFN-1b was a substrate for TYK2. Results reported here (Fig. 3) demonstrate that catalytically active TYK2 is required for IFN-β-mediated phosphorylation of IFNAR-1 in intact cells. However, phosphorylation of IFNAR-2 by IFNs was not dependent on kinase activity of TYK2. Further, despite the absence of IFNAR-1 phosphorylation in U1.KR930 cells, we did see transcriptional induction of IFN-stimulated genes although the levels were lower than those for the wild-type U1.wt cells (10). Mutation analysis of type I IFNs (26) and detailed structure-function studies of the ligand-binding regions of IFNAR-2 (7) have also shown that IFN-induced transcription does not require a direct interaction of ligand with IFNAR-1.

Catalytically active TYK2 was essential for STAT3 phosphorylation by type I IFNs. Our data suggest that failure to phosphorylate IFNAR-1 abrogates STAT3 phosphorylation in U1.KR930 cells. The requirement for STAT1 and STAT2 to initiate characterization as a transcription factor that mediates transcriptional response to IFN is less certain. STAT3 was initially characterized as a transcription factor that mediates cellular responses to IL-6 and EGF (31). STAT3 is activated by a number of growth factors, oncogenes, and nonmitogenic stimuli, and STAT3-null mice are not viable (32). Recent studies indicated that STAT3 associates with the IFNAR-1 chain of the type I IFN receptor in a tyrosine phosphorylation-dependent manner upon IFN-α addition (11). Over-expression of STAT3 in a Daudi cell line resistant to antiviral and antiproliferative effects of IFNs restored an IFN-sensitive phenotype (13). In our studies, U1.wt and U1.KR930 cells clearly manifested IFN-β-regulated antiviral competence. However, in U1.KR930, growth inhibitory responses to IFN-β were not observed. Therefore, STAT3 activation may be required for induction of genes that mediate antiproliferative effects of type I IFNs in certain cell types. Further insight into the role of STAT3 in IFN signaling and function may be obtained through the identification of IFN-inducible genes whose expression requires activated STAT3. By comparison, P13K activity, readily induced by IFN-β in U1.KR930 cells, does not appear to be sufficient for activation of p85 with Tyk2, Jak1, IFNAR-1, or IFNAR-2c (17). In U1A-derived cells, we found P13K to co-immunoprecipitate with IFNAR-1 in the presence or absence of IFN-β in both U1.wt and U1.KR930 cells (Fig. 4a).

---

3 M. R. S. Rani and R. M. Ransohoff, unpublished observations.

D. W. Leaman, M. R. S. Rani, and R. M. Ransohoff, unpublished observations.
IFN-β-mediated antiproliferative effects in the absence of activated STAT3. Genes whose maximal induction by IFN-β appears to require PI3K action have been reported (12). Our results extend current understanding of the mechanism by which IFN-β activates PI3K in U1A-derived cell lines. Catalytically active JAK1 is known to be required for PI3K activation in response to IFN-α (33). Our studies indicate TYK2 kinase activity is not required for IFN-induced activation of PI3K. We also found that p85 did not co-precipitate with anti-STAT3 nor associate with phosphopeptides derived from STAT3 (data not shown). Rather, we describe a novel pathway by which IFN-β-mediated PI3K activation, through a ligand-independent association between p85 and IFNAR-1. In these cells, PI3K activation by IFN-β proceeded equally in the presence or absence of catalytically active TYK2. This result suggests that TYK2 and PI3K signal downstream in parallel, rather than through an obligate kinase cascade. The physiological function(s) of PI3K activation by IFN-β remain to be defined through experiments in which both TYK2 and PI3K-dependent signaling are blocked.

Acknowledgment—We thank George Stark for advice.

REFERENCES
1. Velazquez, L., Fellous, M., Stark, G. R., and Pellegrini, S. (1992) Cell 70, 313–322
2. Pellegrini, S., John, J., Shearer, M., Kerr, I. M., and Stark, G. R. (1989) Mol. Cell. Biol. 9, 4605–4612
3. Uze, G., Lutfalla, G., and Mogensen, K. E. (1995) J. Interferon Cytokine Res. 15, 3–26
4. Richter, M. F., Dumenil, G., Uze, G., Fellous, M., and Pellegrini, S. (1998) J. Biol. Chem. 273, 24723–24729
5. Velazquez, L., Mogensen, K. E., Barbieri, G., Fellous, M., Uze, G., and Pellegrini, S. (1996) J. Biol. Chem. 271, 20494–20500
6. Gauzzi, M. C., Velazquez, L., McKendry, R., Mogensen, K. E., Fellous, M., and Pellegrini, S. (1996) J. Biol. Chem. 271, 3327–3334
7. Gauzzi, M. C., Velazquez, L., McKendry, R., Mogensen, K. E., Fellous, M., and Pellegrini, S. (1996) J. Biol. Chem. 271, 3327–3334
8. Colamonici, O. R., Uyttendaele, H., Domanski, P., Yan, H., and Pellegrini, S. (1996) Mol. Cell. Biol. 16, 33165–33168
9. Yan, H., Krishnan, K., Greenlund, A. C., Gupta, S., Lim, J. T. E., Schreiber, R. D., Schindler, C. W., and Krolewski, J. J. (1996) EMBO J. 15, 1064–1074
10. Rani, M. R. S., Gauzzi, C., Pellegrini, S., Fish, E., Wei, T., and Ransohoff, R. M. (1999) J. Biol. Chem. 274, 1891–1897
11. Yang, C.-H., Shi, W., Basu, L., Murti, A., Constantinescu, S. N., Blatt, L., Croze, E., Mullersman, J. E., and Pfeffer, L. M. (1996) J. Biol. Chem. 271, 8057–8061
12. Pfeffer, L. M., Mullersman, J. E., Pfeffer, S. R., Murti, A., Shi, W., and Yang, C. H. (1997) Science 276, 1418–1420
13. Yang, C. H., Murti, A., and Pfeffer, L. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5568–5572
14. Fry, D. W., Kraker, A. J., McMichael, A., Ambroso, L. A., Nelson, J. M., Leopold, W. R., Connors, R. W., and Bridges, A. J. (1994) Science 263, 1093–1095
15. Exton, J. H. (1994) Biochim. Biophys. Acta 1212, 26–42
16. Coffer, P., Lutticken, C., van Puijenbroek, A., Kloop-de Jong, M., Horn, F., and Kruijer, W. (1995) Oncogene 10, 985–994
17. Uddin, S., Fish, E. N., Sheer, D. A., Gardziola, C., White, M. F., and Platania, L. C. (1997) J. Immunol. 158, 2390–2397
18. Ghislain, J. J., and Fish, E. N. (1996) J. Biol. Chem. 271, 12408–12413
19. Han, Y., Rogers, N., and Ransohoff, R. (1999) J. Interferon Cytokine Res. 19, 751–760
20. Han, Y., Watling, D., Rogers, N. C., and Stark, G. R. (1997) Mol. Endocrinol. 11, 1180–1188
21. Reddy, S. A., Huang, J. H., and Liao, W. S. (1997) J. Biol. Chem. 272, 29167–29173
22. Croze, E., Russell-Harde, D., Wagner, T. C., Pu, H., Pfeffer, L. M., and Perez, H. D. (1996) J. Biol. Chem. 271, 33165–33168
23. Hayes, T. E., Kitchen, A. M., and Cochran, B. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1272–1276
24. Colamonici, O. R., Uyttendaele, H., Domanski, P., Yan, H., and Krolewski, J. J. (1994) J. Biol. Chem. 269, 3518–3522
25. Uddin, S., Yeunash, L., Sun, X.-J., Sweet, M. E., White, M. F., and Platania, L. C. (1995) J. Biol. Chem. 270, 15938–15941
26. Han, Y., Watling, D., Rogers, N. C., and Stark, G. R. (1997) Mol. Endocrinol. 11, 1180–1188
27. Croze, E., Russell-Harde, D., Wagner, T. C., Pu, H., Pfeffer, L. M., and Perez, H. D. (1996) J. Biol. Chem. 271, 33165–33168
28. Han, Y., Rogers, N., and Ransohoff, R. (1999) J. Interferon Cytokine Res. 19, 751–760
29. Han, Y., Watling, D., Rogers, N. C., and Stark, G. R. (1997) Mol. Endocrinol. 11, 1180–1188
30. Reddy, S. A., Huang, J. H., and Liao, W. S. (1997) J. Biol. Chem. 272, 29167–29173
31. Croze, E., Russell-Harde, D., Wagner, T. C., Pu, H., Pfeffer, L. M., and Perez, H. D. (1996) J. Biol. Chem. 271, 33165–33168
32. Hay, G. M., and Pellegrini, S. (1998) J. Biol. Chem. 273, 8003–8008
33. Leung, S., Qureshi, S. A., Kerr, I. M., Darnell, J. E., Jr., and Stark, G. R. (1995) Mol. Cell. Biol. 15, 1312–1317
34. Qureshi, S. A., Leung, S., Kerr, I. M., Stark, G. R., and Darnell, J. E., Jr. (1996) Mol. Cell. Biol. 16, 288–293
35. Merz, M. A., White, J. M., Sheehan, K. C. F., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D., Carver-Moore, K., Dube, R. N., Clark, R., Agu, M., and Schreiber, R. D. (1996) Cell 84, 431–442
36. Durbin, J. E., Hackenmiller, R., Simon, M. C., and Levy, D. E. (1996) Cell 84, 443–456
37. Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994) Science 264, 95–98
38. Takeda, K., Noguchi, K., Shi, W., Tanaka, T., Matsumoto, M., Yoshida, N., Kishimoto, T., and Akira, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3801–3804
39. Burfoot, M. S., Rogers, N. C., Watling, D., Smith, J. M., Pons, S., Paonessaw, G., Pellegrini, S., White, M. F., and Kerr, I. M. (1997) J. Biol. Chem. 272, 24183–24190