

- 200, 38 (1991); S. S. Taylor *et al.*, *Trends Biochem. Sci.* **18**, 84 (1993).
11. D. R. Knighton *et al.*, *Science* **253**, 407 (1991).
12. H. L. De Bondt *et al.*, *Nature* **363**, 595 (1993).
13. K699 cells were transformed with the CAK1-HA integration vector, pFHE86, containing an 860-base pair fragment of the Cak1 coding region (derived from polymerase chain reaction amplification of genomic DNA) fused to an HA tag (18) followed by an actin terminator, in a vector (pRS305) containing the *LEU2* gene [R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)]. The COOH-terminal Cak1 sequence was thereby changed from EKP to EKE-MAYPYDVPDYASLGPGL (19).
14. R. P. Fisher and D. O. Morgan, unpublished data.
15. R. Y. Poon *et al.*, *J. Cell Sci.* **107**, 2789 (1994); J.-P. Tassan, S. J. Schultz, J. Bartek, E. A. Nigg, *J. Cell Biol.* **127**, 467 (1994); A. J. Brown, T. Jones, J. Shuttleworth, *Mol. Biol. Cell* **5**, 921 (1994).
16. R. Shiekhattar *et al.*, *Nature* **374**, 283 (1995); R. Roy *et al.*, *Cell* **79**, 1093 (1994); H. Serizawa *et al.*, *Nature* **374**, 280 (1995).
17. W. J. Feaver, J. Q. Svejstrup, N. L. Henry, R. D. Kornberg, *Cell* **79**, 1103 (1994).
18. D. Desai, Y. Gu, D. O. Morgan, *Mol. Biol. Cell* **3**, 571 (1992).
19. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; K, Lys; L, Leu; M, Met; P, Pro; S, Ser; V, Val; and Y, Tyr.
20. In a typical CAK preparation, log phase cells of strain K699 were lysed by mechanical disruption in a Bead Beater (BioSpec) in lysis buffer [25 mM Hepes (pH 7.4), 50 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM dithiothreitol (DTT), 5 mM NaF, 5 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μ g/ml), and aprotinin (9 mU/ml)]. Nucleic acids were precipitated with polyethyleneimine, and the lysate was clarified by centrifugation (1 hour, 100,000g). CAK activity in the crude lysate (~11 g of total protein) was precipitated with ammonium sulfate (30 to 60% saturation), resuspended in buffer D [25 mM Hepes (pH 7.4), 1 M $(\text{NH}_4)_2\text{SO}_4$, 10% glycerol, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 5 mM β -glycerophosphate], and loaded onto a 180-ml phenyl-Sepharose column. The column was washed with buffer A [25 mM Hepes (pH 7.4), 10% glycerol, 1 mM EDTA, 1 mM DTT, 5 mM NaF, 5 mM β -glycerophosphate] plus 300 mM $(\text{NH}_4)_2\text{SO}_4$ and then eluted with buffer A. The eluate was loaded on an 80 ml DEAE-Sepharose column and eluted with a linear NaCl gradient in buffer A. Peak fractions were passed through sulphopropyl (SP) Sepharose, loaded onto Cibacron blue Sepharose (8 ml), and eluted with a NaCl gradient in buffer A. Peak fractions were concentrated by ammonium sulfate precipitation and fractionated on a Pharmacia Superdex 200 gel filtration column (125 ml) equilibrated with buffer A (Fig. 1A). Peak fractions were pooled and loaded onto a heparin-Sepharose column (25 ml) and eluted isocratically with buffer A. The eluate was concentrated with a 1-ml Mono Q column, loaded onto a 1-ml Cibacron blue Sepharose column, and eluted with a linear NaCl gradient (Fig. 1B). Figure 1C illustrates the peak CAK fraction from a heparin-Sepharose column in a separate preparation.
21. M. R. Gerber, A. Farrell, R. Deshaies, I. Herskowitz, D. O. Morgan, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4651 (1995).
22. For measurement of CAK activity, purified complexes of human CDK2-HA and a truncated form of human cyclin A (CycA Δ 171) (3) were immobilized on protein A-Sepharose beads coated with monoclonal antibody to HA (12CA5) and incubated for 15 min at 25°C with the desired sample and 10 mM MgCl_2 and 1 mM ATP. Beads were washed once with HBST [10 mM Hepes (pH 7.4), 150 mM NaCl, 0.05% Triton X-100], once with HBS [10 mM Hepes (pH 7.4), 150 mM NaCl], and once with kinase buffer [25 mM Hepes (pH 7.4), 10 mM MgCl_2], and then incubated for 10 min at 25°C with kinase buffer plus 100 μ M ATP, 5 μ g histone H1, and 1 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.
23. Partially purified CAK was precipitated with acetone, subjected to polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The 44-kD component (~300 to 500 ng) was excised, digested with trypsin, and fractionated by reversed-phase high-performance liquid chromatography on a 1.0-mm Reliasil C₁₈ column. The masses of four tryptic peptides were determined by matrix-assisted laser-desorption time-of-flight mass spectrometry [H. Erdjument-Bromage, M. Lui, D. M. Sabatini, S. H. Snyder, P. Tempst, *Protein Sci.* **3**, 2435 (1994); S. Geromanos, P. Casteels, C. Elicone, M. Powell, P. Tempst, in *Techniques in Protein Chemistry*, J. W. Crabb, Ed. (Academic Press, San Diego, CA, 1994), vol. 5, pp. 143–150], and two of these peptides were sequenced by automated Edman degradation [P. Tempst, S. Geromanos, C. Elicone, H. Erdjument-Bromage, *Methods* **6**, 248 (1994)]. Small amounts of two peptides from unrelated proteins were also present in the 44-kD fraction and presumably represent contaminants.
24. Yeast lysates were prepared by mechanical disruption in lysis buffer (20). Lysate (500 μ g) or purified mammalian CAK (CDK7-HA-cyclin H-MAT1, 100 ng) were subjected to immunoprecipitation with 12CA5 as described (21). To analyze CDK phosphorylation, we incubated immunoprecipitates for 10 min at 25°C in kinase assay buffer (22) with 5 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 0.5 μ g of each CDK-cyclin pair (except that 1.5 μ g of GST-Cib2 was used for Cib2 experiments). To analyze CDK activation, we incubated immunoprecipitates for 15 min at 25°C with 500 ng of each CDK-cyclin pair (or 1.5 μ g of GST-Cib2) and 10 mM MgCl_2 , 1 mM ATP, and 5 μ g of HA peptide to reduce binding of HA-tagged substrates to 12CA5 on the beads. To analyze histone H1 activity, we incubated 5 ng (lanes 1 to 6, and 16 to 18) or 50 ng (lanes 7 to 12) of the indicated cyclin-CDK pair for 10 min at 25°C with kinase buffer plus 5 μ g of histone H1 and 1 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.
25. Purified CDK2-HA, CDK2(T160A)-HA, and CycA Δ 171 were prepared as described (3, 18). Cdc28-HA and the mammalian CAK trimer were expressed in Sf9 insect cells with recombinant baculoviruses and purified by conventional chromatography. A GST-Cib2 fusion protein was expressed in bacteria (21) and purified with glutathione affinity resin followed by HiTrap Q-Sepharose.
26. For immunodepletion experiments, 50 μ g of 12CA5 and 100 μ l of protein A-Sepharose were added to 500 μ g of lysate, incubated for 1 hour at 4°C, and removed by centrifugation. Control lysates were incubated with beads alone. To analyze Cak1 protein remaining after depletion, we immunoprecipitated 500 μ g of control or immunodepleted lysate with 12CA5 and analyzed the immunoprecipitates by immunoblotting with antibody to HA (16B12). To assess CAK activity remaining after depletion, we incubated 100 ng of each CDK-cyclin complex for 15 min at 25°C with 50 μ g of lysate (or lysis buffer) and 10 mM MgCl_2 and 1 mM ATP. Activated CDKs were recovered by immunoprecipitation with 12CA5 and tested for histone H1 kinase activity as described (22).
27. We thank R. Fisher, H. Chamberlin, and D. Kellogg for reagents; A. Murray for valuable advice; K. Liu and members of the Morgan laboratory for comments on the manuscript; and M. Lui for assistance with protein structural analysis. Supported by grants (to D.O.M.) from the National Institute of General Medical Sciences, the Markey Charitable Trust, and the Rita Allen Foundation, as well as grants from the NSF (to P.T.) and the National Cancer Institute (to the Sloan-Kettering Sequencing Lab). F.H.E. is supported by a University of California President's fellowship. A.F. is supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada.

23 July 1996; accepted 20 August 1996

Regulation of Interferon- γ -Activated STAT1 by the Ubiquitin-Proteasome Pathway

Tae Kook Kim and Tom Maniatis*

STAT proteins (signal transducers and activators of transcription) are latent cytoplasmic transcription factors that are phosphorylated by Janus kinases in response to cytokines. Phosphorylated STAT proteins translocate to the nucleus, where they transiently turn on specific sets of cytokine-inducible genes. The mechanism that controls the amounts of activated STAT proteins is not understood. STAT1 proteins activated by interferon- γ treatment in HeLa cells were shown to be stabilized by a proteasome inhibitor and ubiquitinated *in vivo*. Thus, the amount of activated STAT1 may be negatively regulated by the ubiquitin-proteasome pathway.

Cytokines regulate many aspects of cellular growth, differentiation, and activation and play a critical role in immune and inflammatory responses. Analysis of the pathways involved in interferon- α (IFN- α) and IFN- γ induction has led to the identification of the Janus kinase (Jak)-STAT pathway of cytokine induction (1). The binding of IFNs to their corresponding cell surface receptors results in the activation of Jak kinases, which phosphorylate STAT proteins. In the case of IFN- γ , phosphorylation of the STAT1 protein leads to STAT1's

translocation into the nucleus, where it activates specific target genes by binding to a regulatory sequence termed GAS (IFN- γ -activated site). The amount of phosphorylated STAT1 protein is maximal between 15 and 30 min after IFN- γ treatment and then rapidly decreases to undetectable levels within 1 to 2 hours (2, 3). This negative regulation of STAT levels is very important because the duration of the IFN response is critical and cells must respond to numerous cytokines simultaneously. Without this regulation, toxic amounts of gene products could accumulate, and cells could not modulate or coordinate various cytokine responses. In this report, we address the possible role of ubiquitin-dependent proteolysis

Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed.

in the negative regulation of STAT1.

To determine whether STAT1 is degraded by a proteasome, we examined the

Fig. 1. The proteasome inhibitor MG132 stabilizes IFN- γ -activated STAT1 DNA binding activity. HeLa cells were incubated with medium containing 0.125% dimethyl sulfoxide (DMSO) (lanes 1 and 4), 50 μ M calpain inhibitor II (CI) (lanes 2 and 5), or 50 μ M MG132 (lanes 3 and 6) for 1 hour. Cells were then treated without (lanes 1 to 3) or with (lanes 4 to 6) IFN- γ for 1 hour. Extracts were prepared and analyzed by an electrophoretic mobility-shift assay with a 32 P-labeled GAS DNA probe (2). STAT1-P, phosphorylated STAT1.

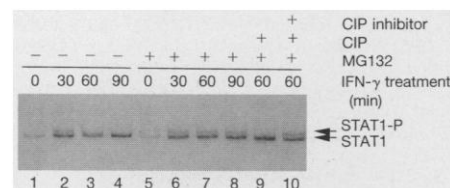


Fig. 2. MG132 preferentially blocks degradation of phosphorylated STAT1 induced by IFN- γ . HeLa cells were incubated with medium containing 0.125% DMSO (lanes 1 to 4) or 50 μ M MG132 (lanes 5 to 10) for 1 hour. The cells were then left untreated (lanes 1 and 5) or treated with IFN- γ for 30 (lanes 2 and 6), 60 (lanes 3, 7, 9, and 10), or 90 min (lanes 4 and 8). Lanes 9 and 10 contained calf intestinal phosphatase (CIP) with and without phosphatase inhibitor mixture (13), respectively. Degradation of STAT1 was followed by an immunoblot analysis with STAT1-specific antibodies.

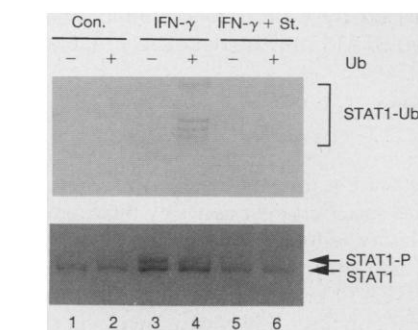


Fig. 3. IFN- γ induces phosphorylation and ubiquitination of STAT1 in vivo. HeLa cells were transfected with His₆ (lanes 1, 3, and 6) or His₆-ubiquitin (Ub) (lanes 2, 4, and 6) expression plasmids (6) and then treated with IFN- γ (lanes 3 and 4), IFN- γ plus staurosporine (lanes 5 and 6), or left untreated (control, con.). His₆-tagged proteins were purified from lysates of the transfected cells by nickel chromatography (7) before immunoblot assay with anti-STAT1 (top). (Bottom) The immunoblot probed with anti-STAT1 without purification of His₆-ubiquitin-conjugated proteins.

effect of the proteasome inhibitor MG132 on the amounts of activated STAT1 (4). Because activated STAT1 can interact specifically with the GAS DNA sequence, we used a DNA binding assay for STAT1 activation with extracts from HeLa cells treated with MG132 for 1 hour before IFN- γ treatment (Fig. 1). Treatment of cells with IFN- γ induced STAT1-GAS DNA complex formation (lane 4). The presence of MG132 during IFN- γ treatment significantly increased or stabilized the GAS DNA binding activity of STAT1 (lane 6). In contrast, STAT1 activation was unaffected by the cysteine protease inhibitor calpain inhibitor II (lane 5). These results show that the amount of activated STAT protein is controlled by a process that involves degradation by the proteasome.

To further investigate the role of proteasome in regulating STAT1 activity, we used an immunoblot assay and STAT1-specific antibodies to examine the effect of MG132 on STAT1 protein amounts. We incubated HeLa cells with or without MG132 for 1 hour before IFN- γ treatment and recorded the amount of STAT1 as a function of time (Fig. 2). As previously shown (2), a slower migrating band corresponding to the phosphorylated STAT1 protein (STAT1-P) was observed at the first time point 30 min after induction (lane 2). Interestingly, STAT1-P rapidly disappeared as a function of time after induction, whereas the amount of unphosphorylated STAT1 remained almost constant (lanes 2 to 4). In contrast, MG132

specifically blocked the disappearance of phosphorylated STAT1 (lanes 5 to 8). This result, in conjunction with the DNA binding data of Fig. 1, suggests that the phosphorylated form of STAT1 is preferentially degraded during IFN- γ induction and that this degradation requires the proteasome.

To determine whether activated STAT1 is a direct target for the proteasome, we examined whether STAT1 can be ubiquitinated in vivo, because, in most cases, ubiquitination is required for proteolysis by the 26S proteasome (5). Specifically, we attempted to detect multi-ubiquitinated STAT1 in cells treated with IFN- γ . A hexahistidine-tagged ubiquitin (His₆-ubiquitin) gene was transiently overexpressed in HeLa cells treated with IFN- γ (6) and the His₆-ubiquitin and its conjugates were then purified by nickel chromatography (7). STAT1 was detected by immunoblot analyses with antibodies to STAT1 (anti-STAT1). A ladder of bands corresponding to multi-ubiquitinated STAT1 was clearly detected when His₆-ubiquitin was overexpressed in the HeLa cells treated with IFN- γ (Fig. 3, lane 4). It is interesting that overexpression of His₆-ubiquitin resulted in a slight decrease in the phosphorylated STAT1 protein in IFN- γ -treated cells (Fig. 3; compare lanes 3 and 4). In addition, ubiquitinated STAT1 could not be detected in cells treated with IFN- γ and staurosporine (8), an inhibitor of IFN- γ -dependent phosphorylation of STAT1 protein (lanes 5 and 6). Thus, IFN- γ -dependent phosphorylation of STAT1 appears to be required for its ubiquitination.

Fig. 4. Phosphorylation is important for ubiquitination of STAT1 in IFN- γ -treated cells. (A) HeLa cells were treated with 0.125% DMSO (lane 1) or 50 μ M MG132 (lanes 2 to 5) for 1 hour before addition of DMSO (lanes 2 and 3) or IFN- γ (lanes 4 and 5). Cell extracts were prepared as described (9) 30 (lanes 2 and 4) and 60 min (lanes 3 and 5) after addition of DMSO or IFN- γ . The extracts were then subjected to immunoblot analysis with anti-STAT1. Bottom panel is a shorter exposure of top panel to show the phosphorylation of STAT1 with treatment of IFN- γ . (B) HeLa cells were treated as described for lanes 3 and 5 in (A), except that cell extracts were first immunoprecipitated with an anti-STAT1. The immunoprecipitates were purified and analyzed by an immunoblot assay with anti-ubiquitin. The arrow marked IgG indicates immunoglobulin G heavy chain in the immunoprecipitates. (C) Transfection of U3A cells (11) was done with plasmids expressing His₆-tagged wild-type (WT) or mutant (Y701F-S727A) STAT1. After transfection, the cells were incubated in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of MG132 and then treated with (lanes 3, 4, 7, 8, 11, and 12) or without (lanes 1, 2, 5, 6, 9, and 10) IFN- γ . His₆-STAT1 proteins were purified from extracts (6, 9) and analyzed by immunoblots with STAT1 antibody (top) or phosphotyrosine (P-Tyr) antibody (bottom). Ub, ubiquitin.

To further demonstrate that STAT1 is ubiquitinated upon phosphorylation in vivo, we prepared cell extracts at different times after treatment of HeLa cells with IFN- γ in the presence of MG132, which stabilizes the phosphorylated form of STAT1 (see Fig. 2). STAT1 proteins in prepared extracts (9) were then analyzed by immunoblot assays with anti-STAT1. High molecular weight forms of STAT1 were detected as phosphorylated STAT1 accumulated after stimulation with IFN- γ in the MG132-treated cells (Fig. 4A, lanes 4 and 5). These bands were not readily observed in extracts from HeLa cells treated with MG132 alone (lanes 2 and 3). Evidence that these high molecular weight bands correspond to ubiquitinated STAT1 was provided by the observation that they could be specifically recognized by antibody to ubiquitin (Fig. 4B). Thus, consistent with the data of Fig. 3, these results indicate that IFN- γ induces phosphorylation and ubiquitination of STAT1, and accumulation of ubiquitinated STAT1 in the presence of the proteasome inhibitor strongly suggests that activated STAT1 is degraded by the proteasome.

Phosphorylation of Tyr⁷⁰¹ and Ser⁷²⁷ residues in STAT1 is induced by IFN- γ (10). We confirmed the importance of STAT1 phosphorylation in ubiquitination by testing the phosphorylation-defective double mutant of STAT1 (Y701F-S727A) in which Tyr⁷⁰¹ and Ser⁷²⁷ were substituted by Phe⁷⁰¹ and Ala⁷²⁷ in the in vivo ubiquitination assay (see Fig. 4A). U3A cells (11), which lack STAT1 protein, were transfected with expression plasmids for wild-type or mutant (Y701F-S727A) STAT1. The transfected cells were incubated with MG132 and then treated with IFN- γ . STAT1 and its tyrosine phosphorylation were detected by immunoblot analyses with anti-STAT1 and anti-phosphotyrosine, respectively (Fig. 4C). Ubiquitinated forms of STAT1 were readily detected in cells expressing wild-type STAT1 after treatment of both MG132 and IFN- γ (Fig. 4C, lane 8). In contrast, ubiquitination was greatly reduced in the cells treated with MG132 and IFN- γ that expressed the phosphorylation-defective STAT1 mutant (Fig. 4C, lane 12). Thus, phosphorylation of STAT1 is a prerequisite for its ubiquitination. Consistent with this conclusion, the tyrosine-phosphorylated form of STAT1 was ubiquitinated (Fig. 4C, bottom, lane 8). This regulatory mechanism is reminiscent of the phosphorylation-dependent degradation of G₁ cyclin (12) and of the NF- κ B inhibitor I κ B α (9, 13).

On the basis of these observations, we propose that the amounts of phosphorylated STAT1 protein in IFN- γ -treated cells are, at least in part, controlled by ubiquitin-dependent proteolysis of STAT1. However,

they do not rule out the possibility that the ubiquitin-proteasome pathway functions at other steps in the IFN- γ signal transduction pathway—for example, at the level of the IFN- γ receptor or kinase (14). It also is possible that the amount of activated STAT1 in the nucleus is controlled by dephosphorylation (14). These mechanisms are not mutually exclusive, because multiple control points may be required for stringent regulation of STAT activity.

REFERENCES AND NOTES

1. J. E. Darnell Jr., I. M. Kerr, G. R. Stark, *Science* **264**, 1415 (1994); J. N. Ihle, *Nature* **377**, 591 (1995); T. Taniguchi, *Science* **268**, 251 (1995).
2. K. Shuai, C. Schindler, V. R. Prezioso, J. E. Darnell Jr., *Science* **258**, 1808 (1992).
3. T. K. Kim and T. Maniatis, unpublished data.
4. V. J. Palombella, O. J. Rando, A. L. Goldberg, T. Maniatis, *Cell* **78**, 773 (1994); K. L. Rock *et al.*, *ibid.*, p. 761.
5. S. Jentsch, *Annu. Rev. Genet.* **26**, 177 (1992); A. Hersko and A. Ciechanover, *Annu. Rev. Biochem.* **61**, 761 (1992); A. Ciechanover, *Cell* **79**, 13 (1994); M. Hochstrasser, *Curr. Opin. Cell Biol.* **7**, 215 (1995); A. W. Murray, *Cell* **81**, 149 (1995); A. L. Goldberg, *Science* **268**, 522 (1995); R. J. Deshaies, *Trends Cell Biol.* **5**, 428 (1995).
6. M. Treier, L. M. Staszewski, D. Bohmann, *Cell* **78**, 787 (1994).
7. Cells were lysed in a buffer containing 6 M guanidinium hydrochloride, and ubiquitin-conjugated proteins were purified by nickel chromatography under the denatured condition. These denatured conditions significantly inhibit the deubiquitination activities. Otherwise, it is difficult to detect multi-ubiquitinated proteins because of potent isopeptidase activities that hydrolyze multi-ubiquitin chains.
8. N. Reich and L. M. Pfeffer, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8761 (1990).
9. Z. Chen *et al.*, *Genes Dev.* **9**, 1586 (1995). The extracts were prepared in the presence of SDS (0.1%) and *N*-ethylmaleimide (5 mM) to inhibit potent isopeptidase activities that may affect the detection of multi-ubiquitinated proteins.
10. K. Shuai, G. R. Stark, I. M. Kerr, J. E. Darnell Jr., *Science* **261**, 1744 (1993); Z. Wen, Z. Zhong, J. E. Darnell Jr., *Cell* **82**, 241 (1995).
11. M. Muller *et al.*, *EMBO J.* **12**, 4221 (1993).
12. M. Tyers, G. Tokiwa, R. Nash, B. Futcher, *ibid.* **11**, 1773 (1992); S. R. Salma, K. B. Hendricks, J. Thorne, *Mol. Cell. Biol.* **14**, 7953 (1994); R. J. Deshaies, V. Chau, M. Kirschner, *EMBO J.* **14**, 303 (1995); J. Yaglom *et al.*, *Mol. Cell. Biol.* **15**, 731 (1995).
13. S. Miyamoto, M. Maki, M. J. Schmitt, M. Hatanaka, I. Verma, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12740 (1994); K. Brown, S. Gerstberger, L. Carlson, G. Franzoso, U. Siebenlist, *Science* **267**, 1485 (1995); E. B.-M. Traenkle, *EMBO J.* **14**, 2876 (1995).
14. R. L. Haspel, M. Salditt-Georgieff, J. E. Darnell Jr., *EMBO J.*, in press.
15. We thank G. Stark for the U3A cells, B. Ren and N. Pandey for comments on the manuscript, and J. E. Darnell for discussing unpublished results. Supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation (DRG-1329 to T.K.K.) and NIH grant AI20642 (T.M.).

2 April 1996; accepted 29 July 1996

Regulation of a Neuronal Form of Focal Adhesion Kinase by Anandamide

Pascal Derkinderen, Madeleine Toutant, Ferran Burgaya, Marc Le Bert, Julio C. Siciliano,* Vittorio de Franciscis,† Michèle Gelman, Jean-Antoine Girault‡

Anandamide is an endogenous ligand for central cannabinoid receptors and is released after neuronal depolarization. Anandamide increased protein tyrosine phosphorylation in rat hippocampal slices and neurons in culture. The action of anandamide resulted from the inhibition of adenylyl cyclase and cyclic adenosine 3',5'-monophosphate-dependent protein kinase. One of the proteins phosphorylated in response to anandamide was an isoform of pp125-focal adhesion kinase (FAK+) expressed preferentially in neurons. Focal adhesion kinase is a tyrosine kinase involved in the interactions between the integrins and actin-based cytoskeleton. Thus, anandamide may exert neurotrophic effects and play a role in synaptic plasticity.

Focal adhesion kinase (FAK) is a 125-kD cytosolic tyrosine kinase associated with focal adhesions in nonneuronal cells, where it is phosphorylated in response to integrin

INSERM U 114, Chaire de Neuropharmacologie, Collège de France, 11 place Marcelin Berthelot, 75231 Paris cedex 05, France.

*Present address: Departamento de Histología, Facultad de Medicina, Montevideo, Uruguay.

†On leave of absence from Centro di Endocrinologia ed Oncologia Sperimentale del CNR, Università di Napoli "Federico II," Naples, Italy.

‡To whom correspondence should be addressed. E-mail: girault@infobiogen.fr

engagement and stimulation of various heterotrimeric GTP-binding protein (G protein)-coupled receptors (1). Autophosphorylated FAK recruits kinases of the Src family and triggers signaling cascades similar to those stimulated by growth factor receptors (2). In addition, FAK is enriched in brain, especially in hippocampus, and in young neurons, where it is located in growth cones (3). Phosphorylation of FAK is decreased in knockout mice deficient for the tyrosine kinase Fyn (4), which display abnormal hippocampal development and synaptic plas-