guanosine triphosphate–binding protein (G protein)–coupled receptors. Because the equivalent of the histidine residue investigated in the present study is strictly conserved in all members of this receptor family (*27*), it may be of general functional importance in these G protein–coupled receptors.

REFERENCES AND NOTES

1. M. Jansen, *Z. Orthop. Chir.* **61**, 253 (1934).
2. P. B. Gram, J. L. Fleming, B. Frame, G. Fine, *J. Bone Jt. Surg.* **41A**, 951 (1959).
3. W. H. D. De Haas, W. De Boer, F. Griffioen, *ibid.* **51B**, 290 (1969).
4. J. F. Holt, in *The Clinical Delineation of Birth Defects*, D. Bergsma, Ed. (The National Foundation of the March of Dimes, Baltimore, MD, 1969), vol. 5, pp. 73–75.
5. S. Kikuchi, M. Hasue, M. Watanabe, K. Hasebe, *J. Bone Jt. Surg.* **58B**, 102 (1976).
6. D. S. Rao, B. Frame, W. A. Reynolds, A. M. Parfitt, in *Vitamin D: Basic Research and Its Clinical Application*, A. W. Norman *et al.*, Eds. (de Gruyter, Berlin, 1979), pp. 1173–1176.
7. B. Frame and A. K. Poznanski, in *Pediatric Diseases Related to Calcium*, H. F. DeLuca and C. S. Anast, Eds. (Elsevier, New York, 1980), pp. 269–289.
8. K. Kruse and C. Schütz, *Eur. J. Pediatr.* **152**, 912 (1993).
9. E. Schipani, K. Kruse, H. Jüppner, data not shown.
10. A. B. Abou-Samra *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2732 (1992).
11. F. R. Bringhurst *et al.*, *Endocrinology* **132**, 2090 (1993).
12. C. Lee *et al.*, *ibid.* **135**, 1488 (1994).
13. E. Schipani *et al.*, *ibid.* **132**, 2157 (1993).
14. H. M. Kronenberg *et al.*, in *Handbook of Experimental Pharmacology: Physiology and Pharmacology of Bone*, G. R. Mundy and T. J. Martin, Eds. (Springer-Verlag, Heidelberg, Germany, 1993), pp. 185–201.
15. A. E. Broadus and A. F. Stewart, in *The Parathyroids. Basic and Clinical Concepts*, J. P. Bilzikian, M. A. Levine, R. Marcus, Eds. (Raven, New York, 1994), pp. 259–294.
16. K. Lee *et al.*, *Endocrinology* **134**, 441 (1994).
17. N. Amizuka, H. Warshawsky, J. E. Henderson, D. Goltzman, A. C. Karaplis, *J. Cell. Biol.* **126**, 1611 (1994).
18. A. C. Karaplis *et al.*, *Genes Dev.* **8**, 277 (1994).
19. B. Lanske, A. C. Karaplis, A. Luz, R. C. Mulligan, H. M. Kronenberg, *Bone Miner. Res.* **9** (suppl. 1), 121 (1994).
20. P. R. Robinson, G. B. Cohen, E. A. Zhukovsky, D. D. Oprian, *Neuron* **9**, 719 (1992).
21. T. P. Dryja, E. L. Berson, V. R. Rao, D. D. Oprian, *Nat. Genet.* **4**, 280 (1993).
22. J. Parma *et al.*, *Nature* **365**, 649 (1993).
23. L. Duprez *et al.*, *Nat. Genet.* **7**, 396 (1994).
24. A. Shenker *et al.*, *Nature* **365**, 652 (1993).
25. M. R. Pollak *et al.*, *Nat. Genet.* **8**, 303 (1994).
26. L. S. Robbins *et al.*, *Cell* **72**, 827 (1993).
27. H. Jüppner, *Curr. Opin. Nephrol. Hypertens.* **3**, 371 (1994).
28. E. Schipani *et al.*, *J. Clin. Endocrinol. Metab.*, in press.
29. The H223R mutation was introduced into the plasmid encoding HKrk by oligonucleotide-directed site-specific mutagenesis (*12*) to give HKrk-H223R. The mutation was confirmed by enzymatic digestion with the restriction enzyme Sph I and by nucleotide sequence analysis. Plasmid DNAs encoding the wild-type and the mutant PTH-PTHrP receptor were then transiently expressed in COS-7 cells (*10, 13*).

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Ras-Dependent Induction of Cellular Responses by Constitutively Active Phosphatidylinositol-3 Kinase

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Phosphatidylinositol (PI)-3 kinase is one of many enzymes stimulated by growth factors. A constitutively activated mutant, p110*, that functions independently of growth factor stimulation was constructed to determine the specific responses regulated by PI-3 kinase. The p110* protein exhibited high specific activity as a PI-3 kinase and as a protein kinase. Expression of p110* in NIH 3T3 cells induced transcription from the *fos* promoter. Co-expression of dominant negative Ras blocked this response. When expressed in *Xenopus laevis* oocytes, p110* increased the amount of guanosine 5′-triphosphate–bound Ras, caused activation of the Ras effector Raf-1, and induced Ras-dependent oocyte maturation. These findings show that PI-3 kinase can stimulate diverse Ras-dependent cellular processes, including oocyte maturation and *fos* transcription.

PI-3 kinase activity has been correlated with the actions of growth factors and oncogenes (*1*). Studies with mutants of platelet-derived growth factor (PDGF) receptor have shown that PI-3 kinase is required for PDGF-mediated mitogenic signaling (*2*). In fibroblast cell lines, a mutant PDGF receptor that no longer associated with PI-3 kinase did not activate c-Ras (*3*). Although this observation implies that PI-3 kinase acts upstream of Ras in PDGF-stimulated signaling, the interpretation of this type of experiment is complicated by the possibility that several signaling molecules share the same binding site. To investigate directly whether PI-3 kinase can transmit signals through a Ras-dependent pathway, we generated a form of PI-3 kinase that is constitutively active and does not require receptor stimulation.

The catalytic subunit of PI-3 kinase, p110, exhibits enzymatic activity in mammalian cells only when bound, through its NH_2-terminal region, to the p85 subunit or to a 102–amino acid fragment of the inter–Src homology region 2 (iSH2) region of p85 (*4–6*). We postulated that a molecule composed only of elements of p85 and p110 that are essential for enzymatic activity might be constitutively active. To generate an activated p110 mutant, p110*, we covalently attached the iSH2 region of p85 to the NH_2-terminus of p110. A hinge region composed of a "glycine-kinker" was inserted between iSH2 and p110 to allow the iSH2 domain to more easily interact with the p110 NH_2-terminal domain (Fig. 1). The p110* protein exhib-

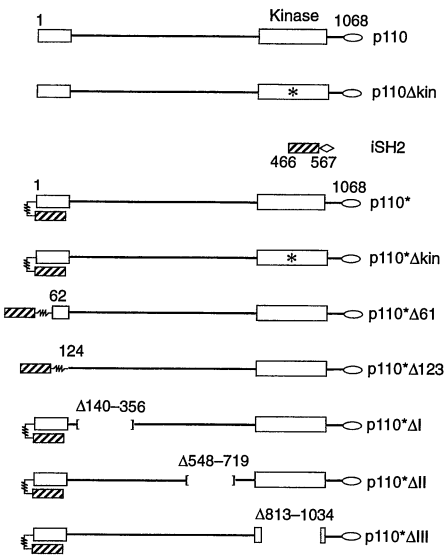

Fig. 1. Schematic representation of the p85 and p110 derivatives used in this study (*16*). The p110 constructs were tagged at the COOH-terminus with the Myc epitope (oval); the iSH2 fragment of p85 contained a COOH-terminal influenza virus hemagglutinin (HA) epitope tag (diamond). The box labeled Kinase represents the p110 region with similarity to the catalytic domain of protein kinases. The domain responsible for the interaction with the iSH2 domain of the p85 subunit is shown as a small box at the p110 NH_2-terminus. The p110Δkin protein is a kinase-deficient version of p110 in which the ATP-binding site was mutated (*16*), as indicated by an asterisk within the catalytic domain. The iSH2 domain of p85 that is required for catalytic activity is represented by a striped bar. The first and last amino acids of fragments are numbered with respect to their position in the wild-type p85 or p110 sequence. The p110* protein is a constitutively active chimera that contains the iSH2 domain of p85 fused to the NH_2-terminus of p110 by means of a flexible glycine-kinker (*16*). Plasmid p110*Δkin is the kinase-deficient version of p110*. Plasmids p110*Δ61 and p110*Δ123 lack 61 or 123 amino acids, respectively, from the p110 NH_2-terminus and can no longer associate with iSH2 (*5*). Plasmids p110*ΔI, p110*ΔII, and p110*ΔIII are mutants that have internal deletions within the p110 structure as indicated (*16*).

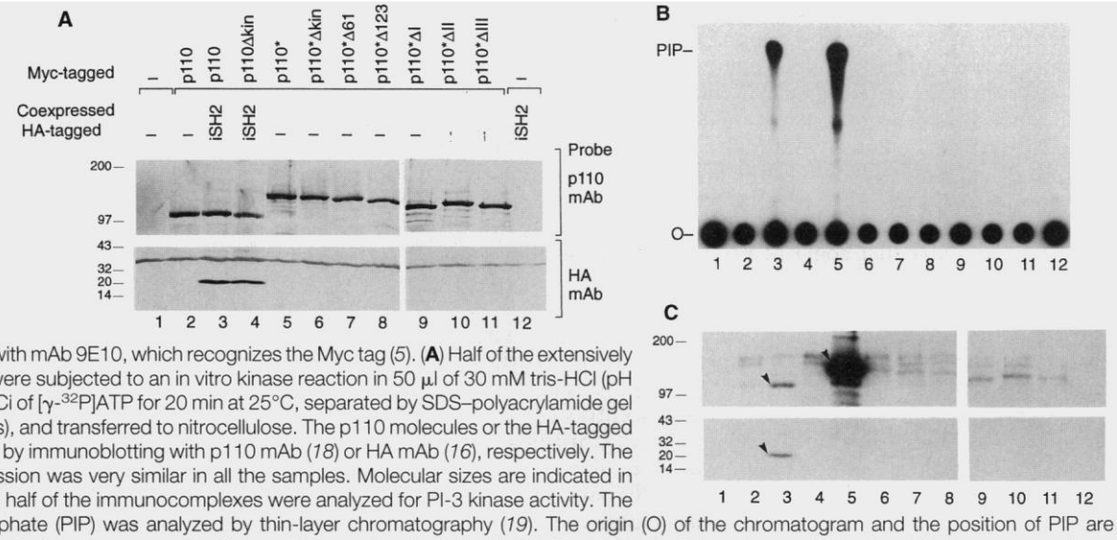

Fig. 2. Functional characterization of the constitutively active p110* and its derivatives. All p110 derivatives were transiently expressed in COS cells (*17*). Wild-type p110 and its kinase-deficient version, p110Δkin, served as controls and were expressed alone (–) or expressed with the iSH2 fragment of p85 as indicated. Myc-tagged p110 or p110* molecules were immunoprecipitated from cell lysates with mAb 9E10, which recognizes the Myc tag (*5*). (**A**) Half of the extensively washed immunocomplexes were subjected to an in vitro kinase reaction in 50 μl of 30 mM tris-HCl (pH 7.5), 10 mM $MnCl_2$, and 10 μCi of [γ-^{32}P]ATP for 20 min at 25°C, separated by SDS–polyacrylamide gel electrophoresis (7 to 12% gels), and transferred to nitrocellulose. The p110 molecules or the HA-tagged iSH2 fragment were detected by immunoblotting with p110 mAb (*18*) or HA mAb (*16*), respectively. The level of p110 or p110* expression was very similar in all the samples. Molecular sizes are indicated in kilodaltons. (**B**) The remaining half of the immunocomplexes were analyzed for PI-3 kinase activity. The production of [^{32}P]PI-3 phosphate (PIP) was analyzed by thin-layer chromatography (*19*). The origin (O) of the chromatogram and the position of PIP are indicated. The numbers below the lanes correspond to those of the samples shown in (A). (**C**) The presence of phosphoproteins in the p110 immune complexes was visualized by autoradiography of the gel shown in (A). The positions of phosphorylated p110, iSH2, and p110* are indicated by arrowheads. Weaker signals resulted from a nonspecific protein kinase that was present in the immunoprecipitates.

ited reproducibly higher specific PI-3 kinase activity than did wild-type p110 coexpressed with iSH2 (Fig. 2, B and C), perhaps because in p110* an intramolecular interaction between iSH2 and p110 is kinetically favored. As with PI-3 kinase activity, the protein kinase activity of p110, measured in an autophosphorylation assay, was dependent on its association with iSH2 (Fig. 2C). Kinase-deficient versions of p110 that were mutated within the putative adenosine triphosphate (ATP)–binding site exhibited neither PI-3 kinase nor protein kinase activity. Mutants of p110 that lacked the NH_2-terminal binding site for iSH2 did not bind iSH2 and were not activated by iSH2 (*5*). Similarly, p110*Δ61 and p110*Δ123, in which iSH2 was fused to p110 molecules with deletions at the NH_2-terminus, exhibited neither PI-3 kinase nor protein kinase activity (Fig. 2, B and C). This finding indicates that the NH_2-terminus of p110 not only functions to tether iSH2 to the remainder of p110, but also has an intrinsic function in regulating catalytic activity.

To investigate the role of PI-3 kinase in growth factor–mediated induction of c-*fos* transcription, we coexpressed p110* in NIH 3T3 cells with pfos-luc, a reporter vector in which the expression of luciferase is controlled by the *fos* promoter. Expression of p110* in the presence of pfos-luc resulted in an increase in luciferase activity when compared to that in control reactions (Fig. 3A). This activity was potentiated by coexpression of c-Ras. With v-Ras there was no potentiation of the ability of p110* to activate the *fos* promoter (*7*). The activation of the *fos* promoter by p110* was completely inhibited by coexpression of dominant negative (DN)–Ras (Fig. 3A); this observation implies that any p110*-mediated increase in *fos* promoter activity is dependent on the Ras pathway. A dominant negative inhibitor of Raf-1 function, NAF (*8*), also blocked p110*-induced activation of the *fos* promoter (Fig. 3A), consistent with the action of Raf-1 as an immediate downstream effector of Ras. The p110* mutants that exhibited neither PI-3 kinase nor protein kinase activity (Fig. 2) also were severely impaired in activating the *fos* promoter (*7*). This result suggests that the kinase activities of p110 are necessary for *fos* induction. To test the effect of wild-type PI-3 kinase on the *fos* promoter, we cotransfected expression vectors encoding p85 and p110 with pfos-luc into NIH 3T3 cells. In contrast to p110*, the wild-type p85–p110 complex did not activate the *fos* promoter unless it was coexpressed with c-Ras (Fig. 3B).

To investigate whether PI-3 kinase could activate the Ras pathway in another system, Myc-tagged p110* was transiently expressed in *Xenopus* oocytes by RNA injection. In oocytes expressing p110*, the amount of guanosine triphosphate (GTP)–bound Ras (Ras·GTP) was more than twice that in control oocytes (Fig. 4A). Ras activation was accompanied by activation of the downstream effectors Raf-1 and Erk, as indicated by the retarded gel electro-

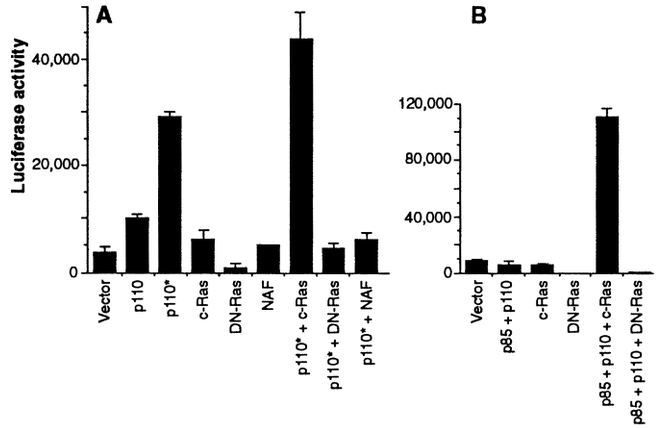

Fig. 3. Activation of c-*fos* promoter by p110*. (**A**) NIH 3T3 cells were transfected with reporter plasmid pfos-luc and vectors expressing p110*, c-Ras, DN-Ras, or NAF (*20*). The amount of expression of p110* was not affected by coexpression of c-Ras, DN-Ras, or NAF (*7*). Luciferase activity was assayed in samples from the cell lysates obtained 48 hours after transfection (*21*). Each bar represents the mean of triplicate samples ± SD. (**B**) NIH 3T3 cells were transfected with pfos-luc and with the indicated combinations of expression vectors.

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phoretic mobility of those proteins (Fig. 4B). Consistent with the known ability of activated forms of Ras to induce maturation (9), oocytes expressing p110* matured, whereas oocytes expressing a catalytically inactive mutant of p110 did not (Fig. 4C). This observation correlated with the finding that only lysates of oocytes injected with p110* RNA exhibited PI-3 kinase activity that could be precipitated by an antibody that recognized the Myc tag (10). Coexpression of DN-Ras decreased the degree of p110*-induced oocyte maturation (10). This set of experiments showed that expression of p110* in *Xenopus* oocytes leads to an increase in the amount of Ras·GTP, which mediates activation of Raf and Erk and maturation of the oocytes. Our results strongly suggest that PI-3 kinase can act upstream of Ras in this system.

The mechanism by which PI-3 kinase regulates mitogenesis is unknown. Yeast strains deficient in PI-3 kinase do not grow normally (11). Mutant receptors or PI-3 kinase inhibitors have been used to examine PI-3 kinase function. For example, mammalian cells transfected with receptor molecules that do not bind PI-3 kinase are defective in growth factor–stimulated mitogenesis, receptor internalization, and cell migration (2, 12). Inhibitors of PI-3 kinase impair activation of pp70 S6 kinase (13). However, in any study that uses receptor mutants, it is possible that signaling pathways other than those mediated by PI-3 kinase may be affected by the mutations. Moreover, the specificity of PI-3 kinase inhibitors may be in question, particularly because a number of p110 homologs have been discovered recently (14).

Until now it has not been possible to examine the action of PI-3 kinase directly, because the only way to activate PI-3 kinase required the use of tyrosine kinases that bind, phosphorylate, and localize PI-3 kinase. Here, we expressed in cells a PI-3 kinase that was constitutively active in a growth factor–independent manner and showed that PI-3 kinase can activate two pathways: one that is important for the mitogenic response to most growth factors in mammalian cells and the other for *Xenopus* oocyte maturation. An elevated level of GTP-bound Ras in response to p110* expression in oocytes was direct evidence that PI-3 kinase can activate the Ras pathway. The recent finding that PI-3 kinase and Ras form a complex (15) suggests an intimate relation between the functions of these molecules. Our approach should be useful for further studies elucidating how PI-3 kinase triggers this pathway and how this mechanism influences other cellular responses that are activated after growth factor exposure.

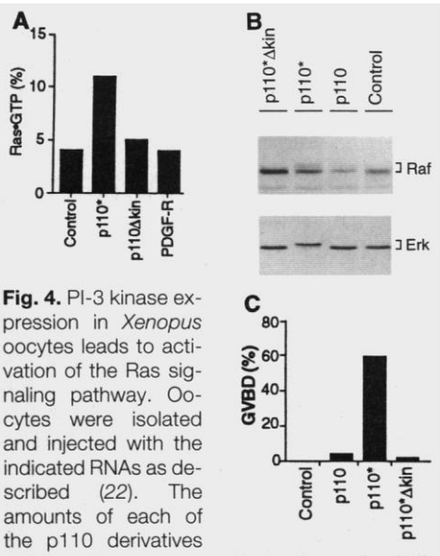

Fig. 4. PI-3 kinase expression in *Xenopus* oocytes leads to activation of the Ras signaling pathway. Oocytes were isolated and injected with the indicated RNAs as described (22). The amounts of each of the p110 derivatives expressed were comparable in all samples (10). Each panel shows results of a representative experiment. Each experiment was performed at least twice, and at least 45 oocytes were injected per condition. PDGF-R, PDGF receptor. (**A**) Ras·GTP amounts were determined as described (23). The oocytes were labeled by 24-hour exposure to 0.5 mCi of [^{32}P]orthophosphate per sample. The percentage of Ras in the active GTP-bound form was determined by the ratio of Ras·GTP/(Ras·GDP + Ras·GTP), where GDP is guanosine diphosphate. (**B**) Oocyte lysates were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to Raf or Erk (22) to investigate the activation state of downstream effectors of Ras. (**C**) Oocyte maturation was scored 24 hours after injection and is shown as percent germinal vesical breakdown (GVBD).

REFERENCES AND NOTES

1. L. Cantley *et al.*, *Cell* **64**, 281 (1991).
2. W. J. Fantl *et al.*, *ibid.* **69**, 413 (1992); M. Valius and A. Kazlauskas, *ibid.* **73**, 321 (1993).
3. T. Satoh, W. J. Fantl, J. A. Escobedo, L. T. Williams, Y. Kaziro, *Mol. Cell. Biol.* **13**, 3706 (1993); W. J. Fantl and L. T. Williams, unpublished results.
4. A. Klippel, J. A. Escobedo, Q. Hu, L. T. Williams, *Mol. Cell. Biol.* **13**, 5560 (1993).
5. A. Klippel, J. A. Escobedo, M. Hirano, L. T. Williams, *ibid.* **14**, 2685 (1994).
6. P. Hu and J. Schlessinger, *ibid.*, p. 2577; K. H. Holt, A. L. Olson, W. S. Moye-Rowley, J. E. Pessin, *ibid.*, p. 42; R. Dhand *et al.*, *EMBO J.* **13**, 511 (1994).
7. Q. Hu, A. Klippel, L. T. Williams, unpublished data.
8. A. M. MacNicol, A. J. Muslin, L. T. Williams, *Cell* **73**, 571 (1993).
9. M. Trahey and F. McCormick, *Science* **238**, 542 (1987).
10. A. J. Muslin, A. Klippel, L. T. Williams, unpublished data.
11. P. V. Schu *et al.*, *Science* **260**, 88 (1993).
12. M. Joly, A. Kazlauskas, F. S. Fay, S. Corvera, *Science* **263**, 684 (1994); V. Kundra *et al.*, *Nature* **367**, 474 (1994).
13. B. Cheatham *et al.*, *Mol. Cell. Biol.* **14**, 4902 (1994); J. Chung, T. C. Grammer, K. P. Lemon, A. Kazlauskas, J. Blenis, *Nature* **370**, 71 (1994).
14. J. Kunz *et al.*, *Cell* **73**, 585 (1993); R. Cafferkey *et al.*, *Mol. Cell. Biol.* **13**, 6012 (1993); E. J. Brown *et al.*, *Nature* **369**, 756 (1994); D. M. Sabatini, H. Erdjument-Bromage, M. Lui, P. Tempst, S. H. Snyder, *Cell* **78**, 35 (1994).
15. P. Rodriguez-Viciana *et al.*, *Nature* **370**, 527 (1994).
16. Vectors directing the expression of HA-tagged iSH2 or Myc-tagged p110 have been described (4, 5). HA-tagged molecules were recognized by the murine monoclonal antibody (mAb) to influenza virus HA 12CA5 [I. A. Wilson *et al.*, *Cell* **37**, 767 (1984)]. Myc-tagged p110 derivatives were detected with murine Myc mAb 9E10 [G. Evan, G. Lewis, G. Ramsay, J. M. Bishop, *Mol. Cell. Biol.* **5**, 3610 (1985)]. We obtained the plasmid pCG-p110ΔKin·myc from pCG-p110·myc (5) by deleting nucleotides coding for the ATP-binding motif within the p110 kinase domain with restriction endonucleases Bsr FI and Mae II. The expressed protein lacks amino acids 917 to 950 of wild-type p110. Expression vectors encoding chimeric p110*, p110*Δ61, and p110*Δ123 molecules (Fig. 1) were derived from described constructs (5). A fragment encoding the p85 iSH2 domain was fused in-frame at its COOH-terminus to the glycine-kinker region in vector pGEX-KG [K. L. Guan and J. E. Dixon, *Anal. Biochem.* **192**, 262 (1991)] and then ligated with Xba I ends to the NH_2-terminus of p110. The glycine-kinker contains seven glycine residues and has been successfully used as a flexible hinge region. The plasmid p110*ΔI lacks the Bst BI fragment of the mouse p110 coding region; in p110*ΔII an Asp 700–Afl II fragment was deleted; and in p110*ΔIII a large portion of the presumed p110 kinase region (between Hpa I and Hind III) was deleted. The corresponding amino acid deletions in these mutant proteins are shown in Fig. 1.
17. COS-7 cells were transfected with the DEAE-dextran method and lysed as described in (5).
18. Murine p110 mAbs E2A, H1A, and I1A were used as a mixture. They were raised against a purified fragment of mouse p110 (amino acids 575 to 1068) expressed in *Escherichia coli*. The epitope recognized by all three mAbs is outside of the ATP-binding site in the p110 kinase domain.
19. D. R. Kaplan *et al.*, *Cell* **50**, 1021 (1987); M. Whitman, C. P. Downes, M. Keeler, T. Keller, L. Cantley, *Nature* **332**, 644 (1988).
20. Plasmid pfos-luc, in which the expression of luciferase is controlled by the c-*fos* promoter, was obtained by replacing the coding region for *CAT* of pFC700 [T. M. Fish, R. Prywes, R. G. Roeder, *Mol. Cell. Biol.* **7**, 3490 (1987)] with the DNA fragment encoding luciferase from pGL2 (Promega). The plasmid pBJ-c-Ras was constructed by digestion of pV-IKS-c-Ras with Eco RI and Bgl II. The *ras* insert was then ligated into vector pBJ downstream of the SRα promoter [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)]. The DN-*ras* fragment was isolated from pBSK-DN-Ras after Xho I and Not I digestion and was then ligated into pBJ DNA. The NAF-encoding DNA fragment (7) was cloned into vector pCG (5).
21. Transient transfection of NIH 3T3 cells with the indicated expression vectors was done with Lipofectamin (Gibco-BRL) according to the manufacturer's instructions. Cells were lysed after 48 hours of incubation in serum-free medium. Luciferase activity assays were performed with a luminometer.
22. A. J. Muslin, A. M. MacNicol, L. T. Williams, *Mol. Cell. Biol.* **13**, 4197 (1993).
23. T. Satoh, M. Endo, M. Nakafuku, S. Nakamura, Y. Kaziro, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5993 (1990).

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