Regulation of Protein Kinase B and Glycogen Synthase Kinase-3 by Insulin and β-Adrenergic Agonists in Rat Epididymal Fat Cells

ACTIVATION OF PROTEIN KINASE B BY WORTMANNIN-SENSITIVE AND -INSENSITIVE MECHANISMS*

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Previous studies using L6 myotubes have suggested that glycogen synthase kinase-3 (GSK-3) is phosphorylated and inactivated in response to insulin by protein kinase B (PKB, also known as Akt or RAC) (Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovic, M., and Hemmings, B. A. (1995) Nature 378, 785–789). In the present study, marked increases in the activity of PKB have been shown to occur in insulin-treated rat epididymal fat cells with a time course compatible with the observed decrease in GSK-3 activity. Isoproterenol, acting primarily through β1-adrenoreceptors, was found to decrease GSK-3 activity to a similar extent (approximately 50%) to insulin. However, unlike the effect of insulin, the inhibition of GSK by isoproterenol was not found to be sensitive to inhibition by the phosphatidylinositol 3'-kinase inhibitors, wortmannin or LY 294002. The change in GSK-3 activity brought about by isoproterenol could not be mimicked by the addition of permeant cyclic AMP analogues or forskolin to the cells, although at the concentrations used, these agents were able to stimulate lipolysis. Isoproterenol, but again not the cyclic AMP analogues, was found to increase the activity of PKB, although to a lesser extent than insulin. While wortmannin abolished the stimulation of PKB activity by insulin, it was without effect on the activation seen in response to isoproterenol. The activation of PKB by isoproterenol was not accompanied by any detectable change in the electrophoretic mobility of the protein on SDS-polyacrylamide gel electrophoresis. It would therefore appear that distinct mechanisms exist for the stimulation of PKB by insulin and isoproterenol in rat fat cells.

Glycogen synthase kinase-3 (GSK-3)1 was first discovered by virtue of its ability to phosphorylate and inactivate the regulatory enzyme of glycogen synthesis in mammals, glycogen synthase (GS) (reviewed in Refs. 1–3). In a number of tissues GS is activated by insulin, and in skeletal muscle this has been shown to involve the dephosphorylation of the sites phosphorylated by GSK-3 (4–7). This suggested that GSK-3 might be inactivated by insulin, although more attention was initially focused on the regulation (activation) of the relevant phosphatase, a glycogen-bound form of protein phosphatase-1 (PP-1G) (8). Recently, however, it has become clear that GSK-3 itself is indeed subject to acute regulation, being inactivated in response to insulin or growth factors (9–16) and also following T-cell stimulation (17). Where tested, this inactivation was reversed by treatment with serine/threonine phosphatases indicating that it was due to increased phosphorylation of GSK-3 on one or more Ser/Thr residues (11).

It has since been shown that the inactivation of GSK-3 is associated with increased phosphorylation of a serine residue near the N terminus (Ser-9) of the β isoform of mammalian GSK-3 which corresponds to Ser-21 of the α isoenzyme (11, 14, 16). This is of special interest since several insulin/growth factor-activated protein kinases have been shown to phosphorylate these residues in GSK-3 in vitro. These include the p90 ribosomal S6 protein kinase (p90rsk), which is downstream of the mitogen-activated protein kinase (MAP kinase) cascade, and the p70 ribosomal S6 kinase (p70S6k) (18, 19). Studies employing rapamycin, which selectively blocks activation of p70S6k, appear to rule out a role for this enzyme in regulating GSK-3 in the cell types thus far studied (10, 12, 13). The evidence for a role for the MAP kinase pathway is mixed, with transfection approaches (either using p90rsk itself (14) or dominant negative mutants of an upstream kinase (15)) providing evidence for a role for this pathway and recent data from fat cells (13) and L6 myotubes (16) giving, respectively, correlative and strong evidence against a role for the MAP kinase pathway in regulating GSK-3 activity in response to insulin. The latter studies (16) provided evidence that protein kinase B (PKB, also known as RAC or Akt) might be responsible for the control of GSK-3 in L6 myotubes. PKB phosphorylates Ser-9 and Ser-21 in the β- and α-isoforms of GSK-3, respectively, is activated by insulin (16, 20, 21), and may lie downstream of phosphatidylinositol 3'-kinase (PI 3'-kinase) (16, 22). PI 3'-kinase is implicated in the control of GSK-3 in several cell types by the observation that wortmannin, a selective but not absolutely specific inhibitor of PI 3'-kinase, blocks the inactivation of GSK-3 by insulin and other agents (10, 12, 13, 16). LY 294002, a structurally unrelated inhibitor of PI 3'-kinase (23), has recently been shown to block the insulin-induced inactivation of GSK-3 in L6 myotubes (16).

In this study we demonstrate that insulin activates PKB in rat fat cells and that this may underlie the effects of insulin on GSK-3 activity in these cells. In addition, we report that the
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**EXPERIMENTAL PROCEDURES**

**Materials**—Male Wistar rats were fed ad libitum up to the time of killing on a stock laboratory diet (CRM; Bioshore, Manen, Cambes, UK). Collagenase was purchased from Worthington Diagnostic Systems (Freehold, NJ). Enhanced chemiluminescence Western blotting detection kits and [γ-32P]ATP were from Amersham Int. (Amersham, Bucks, UK), and pepstatin, leupeptin, and antipain were from Cambridge Research Biochemicals (Harston, Cambes, UK). Microcystin, dithiothreitol, and rapamycin were obtained from Calbiochem (Nottingham, UK). LY 294002 was purchased from the Alexis Corp. (Nottingham, UK). All other chemicals and biochemicals were from Sigma or BDH (both of Poole, Dorset, UK). The β-adrenergic agonist, BRL 37344, was a gift from Dr. P. Young of SmithKline Beecham Pharmaceuticals (Welwyn, Herts, UK). The PKB antisera was raised in rabbits immunized with a synthetic peptide corresponding to residues 465–480 of the human PKB sequence and was used without further purification. Anti-GSK-3 serum was a gift from Dr. J. Vandenheede (Katholieke Universiteit, Leuven, Belgium).

**Preparation and Incubation of Epididymal Fat Cells**—Adipocytes were isolated from epididymal fat pads, preincubated, and then incubated (at 150–250 mg cell dry weight/ml) as described previously (13, 16). For the measurement of PKB activity, fat cells treated with wortmannin, LY 294002, or rapamycin, were incubated (at 150–200 mg dry cell weight/ml) as described previously (13). Adipocytes (150–200 mg dry cell weight) were extracted in 1 ml of 50 mM Hepes (pH 7.6), 0.2 mM EDTA, 2.2 mM EGTA, 1 mM dithiothreitol, 100 mM KCl, 10% glycerol, 1% Triton X-100, 1 μM micropyrin and 5 μg/ml each of pepstatin, leupeptin, and antipain. PKB was then immunoprecipitated from 750 μl of cell extracts with 5 μg of protein A-Sepharose and 5 μl of PKB antisera for 2 h at 4 °C. Immunoprecipitates were washed twice with 1 ml of PKB assay buffer (20 mM MOPS (pH 7.0), 1 mM EDTA, 1 mM EGTA, 0.01% Brij 35, 5% glycerol) containing 0.5 M NaCl and twice with 1 ml of the same buffer without added salt. The protein A beads were finally resuspended in 40 μl of PKB assay buffer containing 0.1% mercaptoethanol and 2.5 mM cAMP-dependent protein kinase inhibitor peptide (IP60). The activity of PKB in these immunoprecipitates was measured using either myelin basic protein (MBP, 0.5 mg/ml final concentration) or the synthetic peptide based on the sequence surrounding the serine phosphorylation site of PKB (Ser-21 of PKB-α and Ser-9 of PKB-β), “cross-tide” (16) (100 μM), for 15 min at 30 °C.

Anion Exchange Chromatography, Gel Electrophoresis, and Immunoblotting of PKB—To separate proteins by anion exchange chromatography, fat cells were extracted as above and applied to a Pharmacia SMART system Mono Q column equilibrated with Buffer A (50 mM Tris (pH 7.0), 0.2 mM EDTA, 2 mM EGTA, 5% glycerol, 0.03% Brij 35 (w/v), 0.1 mM mercaptoethanol, and 1 μg/ml pepstatin, leupeptin, and antipain). The column was developed with a 0–50% gradient of Buffer B (Buffer A plus 1 M NaCl) at a flow rate of 50 μl/min. Fractions (100 μl) were assayed for cross-tide kinase activity (10-μl fraction in a final reaction volume of 25 μl).

Western blotting was performed using immunoprecipitates run on 16-cm SDS-10% polyacrylamide gels (25) and blotted onto Immobilon-P

**FIG. 1.** Time course for the effect of insulin and isoproterenol of GSK-3. Adipocytes were incubated with insulin (83 nM, ○) or isoproterenol (1 μM, ●) for the times indicated prior to extraction. Results are expressed as a percentage of the control value at time 0 (39.5 ± 4.9 pmol of phosphate incorporated into substrate peptide/min/g dry cells, n = 3) and are means ± S.E. for three separate cell preparations. In the absence of hormones the activity of GSK-3 did not alter significantly with time and was 39 ± 3.2 pmol of phosphate/min/g dry cells (n = 3) after the 40-min incubation.

β-adrenergic agonist, isoproterenol, also causes the stimulation of PKB and inactivation of GSK-3 in fat cells. The effects of isoproterenol on these two kinases do not appear to be mediated by increases in the intracellular level of cAMP. A number of lines of evidence suggest that distinct mechanisms are involved in the activation of PKB by insulin and isoproterenol.

**FIG. 2.** The effect of β-adrenergic agonists and agents that increase intracellular cAMP levels on GSK-3 activity. A, adipocytes were incubated for 10 min with 83 nM insulin (Ins), 1 μM isoproterenol (Iso), 5 μM BRL37344 (BRL), 2 μM cpt-cAMP (cpt), 5 mM db-cAMP (db), or 5 μM forskolin (For) prior to extraction. Where indicated 10 μM propranolol (Pro) was added 10 min before addition of isoproterenol. Results are expressed as a percentage of the control value (no additions) for GSK-3 activity (45.6 ± 3.0 pmol of phosphate incorporated into substrate peptide/min/g dry cells, n = nine separate cell preparations) and are means ± S.E. for the number of separate cell preparations shown in parentheses. Significance, as assessed by the Student's t-test, is indicated as follows: ***, p < 0.0001; *, p < 0.001 versus appropriate control. B, dose response for the effect of BRL37344 on GSK-3 activity. Adipocytes were incubated for 10 min with varying concentrations of BRL37344. Results are expressed as a percentage of the control value in the absence of BRL37344 (see A) and are means ± S.E. for two separate cell preparations.
Adipocytes were incubated with effectors as shown for 10 min prior to extraction. Extracts were assayed for GS activity in the presence or absence of glucose 6-phosphate, while the cell incubation media were retained for glycerol assays. Results are shown as the average measurement determined from the number of separate cell preparations shown in parentheses. Where appropriate (for n > 2), values are ± S.E., and significance (p < 0.01 versus control) is assessed by Student’s t test and is denoted by ∗.

### TABLE I

**The effect of various agents on lipolysis and glycogen synthase activity ratio**

| Condition                        | Rate of lipolysis (nmol glycerol released/10 min/g dry cells) | GS activity ratio (−/+)-glucose 6-phosphate |
|----------------------------------|---------------------------------------------------------------|------------------------------------------|
| Control                          | 207 ± 29 (6)                                                  | 0.048 ± 0.009 (6)                        |
| Insulin (83 nm)                  | 225 ± 47 (6)                                                  | 0.238 ± 0.048 (6)∗                       |
| Isoproterenol (1 μM)             | 2495 ± 340 (6)∗                                               | 0.059 ± 0.008 (6)                        |
| Insulin + isoproterenol          | 2833 (2)                                                      | 0.113 (2)                                |
| BRL37344 (5 nm)                  | 1393 (2)                                                      | 0.030 (2)                                |
| cpt-cAMP (2 nm)                  | 1648 (2)                                                      | 0.063 (2)                                |
| db-cAMP (5 nm)                   | 2512 (2)                                                      | 0.055 (2)                                |
| Forskolin (5 nm)                 | 2425 (2)                                                      | 0.057 (2)                                |

Results

**Inactivation of GSK-3 in Fat Cells in Response to Isoproterenol—** Incubation of adipocytes with isoproterenol led to a rapid decrease in the activity of GSK-3 (Fig. 1). Maximum inactivation of GSK-3 in response to isoproterenol was seen after approximately 20 min and was sustained for at least 40 min. This time course and the extent of inactivation (approximately 50%) were broadly similar to the changes in GSK-3 activity seen in response to insulin (see Fig. 1 and Ref. 13), although the effect of insulin was more rapid in onset. The effects of insulin and isoproterenol on GSK-3 activity were not additive (Fig. 2A). GSK-3 activity in all experiments was measured by following phosphate incorporation into a peptide substrate corresponding to the GSK-3 phosphorylation site in eukaryotic initiation factor-2B. A similar inactivation of GSK-3 in response to isoproterenol was also observed when the kinase was assayed using a peptide based on its phosphorylation site in GS (see Ref. 13) (data not shown).

In order to investigate the mechanism by which isoproterenol inactivates GSK-3 in fat cells, a number of agents designed to either mimic or inhibit the effects of β-adrenergic agents was tested. The effects of isoproterenol on fat cells are mediated through its actions on β-adrenergic receptors. However, the effects of isoproterenol on GSK-3 in this system were not blocked by the classical β₁- or β₂-antagonist propranolol (Fig. 2A). This finding suggested that isoproterenol may be acting primarily via the β₂-receptor which is known to be insensitive to propranolol (see Ref. 28). The β₂-receptor is thought to be the primary adrenergic receptor mediating the effects of β-adrenergic agents on lipolysis in rat white adipose tissue (29). Like isoproterenol, the β₂-receptor-specific agonist, BRL 37344 (30, 31), caused a significant decrease in the activity of GSK-3 (Fig. 2A). The effects of BRL 37344 on GSK-3 activity were half-maximal at approximately 5 nm (Fig. 2B). This is consistent with reported EC₅₀ values for the actions of BRL 37344 on the stimulation of lipolysis in this cell type (32–34).

The binding of β-adrenergic agonists to their receptors activates adenylate cyclase and thereby increases cytoplasmic cAMP levels. In order to address the possibility that the inactivation of GSK-3 was a consequence of elevated cAMP, we adopted two approaches. In the first, cells were treated with the cell-permeant cAMP analogues, db-cAMP and cpt-cAMP, which specifically blocks the activation of p70S6k without affecting MAP kinase had no effect upon the inactivation of GSK-3 induced by isoproterenol or BRL 37344 (Fig. 3). Rapamycin also has no effect upon the control of GSK-3 in other cell types tested (10, 12, 13, 16). At the dose used here (20 nm), rapamycin completely blocked the activation of p70S6k induced by insulin (see Ref. 13).

**Insulin and Isoproterenol Activate PKB in Rat Epididymal Fat Cells—**Cross et al. (16) have recently shown that PKB can phosphorylate GSK-3 in vitro and cause its inactivation. PKB phosphorylates the same N-terminal serine residue phosphorylated by p70S6k or p90RSK (18, 19). Since PKB is thought to be

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downstream of PI 3-kinase (20, 22), this would provide an explanation of the ability of wortmannin to block completely the regulation of GSK-3 by insulin and other stimuli. Thus, insulin by, for example, activating PI 3-kinase would also bring about the activation of PKB and therefore the phosphorylation and inactivation of GSK-3.

We have therefore examined the regulation of PKB activity by insulin and isoproterenol in fat cells. Extracts from adipocytes treated with insulin or isoproterenol were separated by Mono Q chromatography essentially as described by Cross et al. (16). Insulin increased the kinase activity toward cross-tide in two broad peaks (I and II) (Fig. 4). Peak I (eluting between 150–250 mM NaCl) was found to coincide with the elution position of PKB as shown by immunoblotting (Fig. 4, inset). The cross-tide kinase activity eluting at this position was thus due, at least in part, to the activity of PKB. Immunoreactive PKB could not be detected in peak II, and thus the activity toward cross-tide in these fractions is likely to be due to some other unidentified kinase. Isoproterenol increased the kinase activity toward cross-tide in peak I but not peak II. The effect of isoproterenol on the kinase activity in this peak was less than the stimulation seen in response to insulin.

The stimulation of PKB by both insulin and isoproterenol in whole cell extracts was confirmed by measuring kinase activity in anti-PKB immunoprecipitates using either cross-tide or myelin basic protein as a substrate. Table II shows that the both hormones increase the activity of PKB, with the effects of insulin being greater than those of isoproterenol. The fold effects observed were similar whether cross-tide or MBP was used to measure kinase activity. To further verify that the kinase activity measured in immunoprecipitates was actually PKB, we preincubated the protein A-Sepharose beads with anti-PKB antibody plus the peptide to which the antiserum was raised (see “Experimental Procedures”) prior to the addition of beads to fat cell extracts. This peptide was able to compete with PKB for binding to the antiserum, as shown by its ability to abolish kinase activity toward MBP in immunoprecipitates from control, insulin-, or isoproterenol-treated cell extracts (data not shown).

The time courses for activation of PKB by insulin and isoproterenol are shown in Fig. 5. Insulin caused an extremely rapid stimulation of PKB activity which declined slowly over 40 min. In contrast, the activation in response to isoproterenol was slower and more transient. A comparison of the time courses for GSK-3 inactivation (Fig. 1) and PKB stimulation (Fig. 5) shows a number of discrepancies between the two. First, complete activation of PKB, even with isoproterenol, was seen after 5 min, while maximal inhibition of GSK-3 occurred only after 20 min. Second, inactivation of GSK-3 was sustained even after PKB activities had returned to basal levels.

The stimulation of PKB by insulin has also been reported in a number of other cell types (16, 20, 21) and is accompanied by a change in the mobility of the protein on SDS-PAGE. This band-shift in response to insulin is thought to be a result of changes in the phosphorylation of PKB (21, 22, 35) and may be seen on stimulation of fat cells with the hormone (see Fig. 6 inset and Ref. 21). Interestingly the increase in PKB activity on treatment with isoproterenol is not associated with any detectable change in the electrophoretic mobility of the protein. The effects of insulin and isoproterenol on PKB activity were not additive (Fig. 6). The secondary effect of isoproterenol again appears to be via the β-adrenergic receptor because BRL 37344 is also able to stimulate PKB. The lack of effect of either cpt-cAMP or db-cAMP indicates that the increase of PKB activity in response to β-adrenergic agonists does not occur as a result of increased cytoplasmic cAMP levels (Fig. 6).

The data in Fig. 7 show that while the effect of insulin was **,** appropriate significance (assessed as in Fig. 2 parentheses the number of separate cell preparations shown in parentheses. Where appropriate significance (assessed as in Fig. 2A) is indicated as follows: **, p < 0.001; *, p < 0.05.

| Condition | MBP kinase activity | Cross-tide kinase activity |
|-----------|---------------------|---------------------------|
| Control   | 0.56 ± 0.04 (10)    | 0.46 ± 0.03 (3)           |
| Insulin   | 1.85 ± 0.13 (17)*   | 2.20 ± 0.32 (3)*          |
| Isoproterenol | 1.15 ± 0.05 (15)*   | 1.24 ± 0.13 (3)*          |
abolished by preincubation of the adipocytes with wortmannin, the inhibitor had no significant effect on the stimulation of PKB by isoproterenol. In agreement with others (21), wortmannin also reversed the effect of insulin on the band-shift seen with PKB on SDS-PAGE (data not shown). Rapamycin was without effect on changes in the activity of PKB in response to either hormone (data not shown).

Given that PKB is reported to lie upstream of p70S6k (22), we determined whether isoproterenol activated this kinase. Isoproterenol gave a significant activation, which was blocked by rapamycin but was modest in comparison to that induced by insulin (Fig. 8). In contrast to the effect of isoproterenol on PKB activation and GSK-3 inhibition, the increase in p70S6k activity was largely eliminated by wortmannin and rapamycin.

**DISCUSSION**

GSK-3 in Rat Epididymal Fat Cells Is Activated by Isoproterenol as Well as Insulin—The data presented in this paper show that in freshly isolated rat adipocytes treated with isoproterenol, there is a marked inhibition of GSK-3 activity. Isoproterenol was also found to increase the activity of the kinase proposed to be upstream of GSK-3, namely PKB. We have been unable to find any change in the activity ratio of GS...
inhibitor was without effect. Isoproterenol has no effect on PKB activation in rat fat cells and suggest that PPI-1G probably plays a more important role in the regulation of glycogen synthesis in vivo. It has been suggested that PPI-1G plays the dominant role in the regulation of rat muscle GS (see Ref. 37). The mechanisms involved in the regulation of glycogen metabolism in different tissues may be distinct. In rat muscle (37) and in 3T3-L1 adipocytes (38) the stimulation of GS by insulin is partially sensitive to inhibition by rapamycin, whereas in primary rat adipocytes it is not (13).

PKB in Rat Epididymal Fat Cells Is Activated by Insulin and Isoproterenol by Apparently Different Mechanisms—As PKB has been implicated as the direct upstream regulator of GSK-3 in L6 myotubes (16), it was important to determine its activity in adipocytes. Here we report the first direct measurement of PKB activity in rat fat cells and have shown substantial and rapid stimulation of the kinase in response to insulin. Isoproterenol was also found to activate PKB in these cells. Overall, the congruence between the actions of all effectors on GSK-3 inactivation and PKB activation supports the view that PKB is the upstream regulator of GSK-3 in fat cells, as concluded by Cross et al. (16) from their studies in L6 myotubes. In further agreement with this view, the activation of PKB in the present study is considerably more rapid than the inactivation of GSK-3 (Figs. 1 and 5). However, in the absence of a specific inhibitor of PKB it is not possible to obtain conclusive proof that PKB is indeed upstream of GSK-3.

The finding that isoproterenol also causes a small stimulation of p70S6K is interesting in light of the proposal that this kinase is downstream of PKB (22). The physiological relevance of the activation of this S6 kinase under these conditions remains to be explained.

Our results also have important implications for understanding the mechanisms by which PKB is regulated in fat cells. The exact means by which PKB activity is controlled remains obscure, with a number of different factors being implicated, acting either independently or in conjunction with each other. In other cell types there is strong evidence that the activation of PKB is mediated via PI 3-kinase (20, 22), although the specific mechanisms involved in the activation of PKB by insulin in fat cells, as concluded by Cross et al. (16) from their studies in L6 myotubes. In further agreement with this view, the activation of PKB in the present study is considerably more rapid than the inactivation of GSK-3 (Figs. 1 and 5). However, in the absence of a specific inhibitor of PKB it is not possible to obtain conclusive proof that PKB is indeed upstream of GSK-3.

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34. Murphy, G. L., Kirkham, D. M., Cawthorne, M. A., and Young, P. (1993) Biochem. Pharmacol. 46, 575–581
35. Andjelkovic, M., Jakubowicz, T., Cron, P., Ming, X.-F., Han, J.-W., and Hemmings, B. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5699–5704
36. Lawrence, J. C., Jr., James, C., and Hiken, J. F. (1986) J. Biol. Chem. 261, 669–677
37. Azpiazu, I., Saltiel, A. R., De Paoli-Roach, A. A., and Lawrence, J. C., Jr. (1996) J. Biol. Chem. 271, 5033–5039
38. Shepherd, P. R., Nave, B. T., and Siddle, K. (1995) Biochem. J. 305, 25–28
39. James, S. R., Downes, C. P., Gigg, R., Grove, S. J. A., Holmes, A. B., and Alessi, D. R. (1996) Biochem. J. 315, 709–713
40. Giorgetti, S., Ballotti, R., Kowalski-Chauvell, A., Cormont, M., and Van Obberghen, E. (1995) Biochem. Biophys. Res. Commun. 216, 526–534
41. Stephens, L., Smrcka, A., Cooke, F. T., Jackson, T. R., Sternweis, P. C., and Hawkins, P. T. (1994) Cell 77, 83–93
42. Thomason, P. A., James, S. R., Casey, P. J., and Downes, C. P. (1994) J. Biol. Chem. 269, 16525–16528
43. Konishi, H., Kuroda, S., Tanaka, M., Matsuzaki, H., Ono, Y., Kameyarna, K., Haga, T., and Kikkawa, U. (1995) Biochem. Biophys. Res. Commun. 216, 526–534
44. Datta, K., Franke, T. F., Chan, T. O. Makris, A., Yang, S.-I., Kaplan, D. R., Morrison, D. K., Golemis, E. A., and Tsichlis, P. N. (1995) Mol. Cell. Biol. 15, 2304–2310